Plasticity of GABAergic Synaptic Inhibition *in vitro* and *in vivo*: From $GABA_A$ Receptor Endocytosis to the Development of Sedative Tolerance to Diazepam

Dissertation

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Colofon:

Cover design: artistic impression of the interneuron depicted on page 27.

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Hoe ver je gaat, heeft met afstand niks te maken
Hooguit met de tijd

Translation:
How far you go, has nothing to do with distance
at the most with time

Bløf (Dutch band)
‘Omarm’ from the album ‘Omarm’

Zusammenfassung

$GABA_A$ Rezeptoren vermitteln den grössten Teil der hemmenden Transmission im Gehirn und sind Zielstrukturen für viele Medikamente, wie z.B. Benzodiazepine, die aufgrund ihrer sedierenden, muskelrelaxierenden, anxiolytischen und antiepileptischen Wirkung verschrieben werden. Bei chronischem Gebrauch entwickelt sich eine Toleranz gegenüber diesen Effekten, wobei der Mechanismus, der zu diesem Wirkungsverlust führt, immer noch unbekannt ist.

In der vorliegenden Arbeit wurden zwei voneinander unabhängige Ansätze verfolgt, um die Regulation der $GABA_A$ Rezeptoren bezüglich der Toleranzentwicklung nach chronischer Diazepam-Behandlung zu untersuchen. Zuerst befassten wir uns mit der konstitutiven Internalisierung von endogenen $GABA_A$ Rezeptoren in kultivierten Neuronen, um zu prüfen, inwiefern dieser Regulationsmechanismus zur Toleranzentwicklung beiträgt. In einem zweiten Schritt konzentrierten wir uns auf Verhaltensexperimente mit Mäusen mit einer Punktmutation, welche spezifische $GABA_A$ Rezeptoren Diazepam-insensitiv macht. Dabei war das Ziel, herauszufinden welche $GABA_A$ Rezeptor Subtypen an der Entwicklung von Toleranz gegenüber der sedativen Wirkung von Diazepam beteiligt sind.

In der ersten Studie benutzten wir immunzytochemische Methoden, um die subzelluläre Verteilung von $GABA_A$ Rezeptoren in der Membran und deren Translokation aufgrund von Internalisierung zu verfolgen. In lebenden hippocampalen Zellen wurden Rezeptoren mit Antikörpern markiert, und zwar unter Bedingungen, die Endozytose erlauben. Oberflächenrezeptoren waren homogen über die Membran verteilt, während die endozytierten Rezeptoren interessanterweise zu deutlich erkennbaren Clustern in einem subsynaptischen Pool akkumulierten, der mit Gephyrin assoziiert war. Da dieser Prozess zeitabhängig war, vermuten wir, dass internalisierte Rezeptoren zu diesem intrazellulären Reservoir umverteilt werden. Die Endozytose erfolgte mit Hilfe von *Clathrin-coated* Vesikeln, denn die Hemmung dieses Mechanismus mit kaltem Puffer, hypertonischer Saccharose oder mit einem Dynamin-inhibitierenden Peptid die Intensität der internalisierten Cluster massiv reduzierte. Die akute Applikation von $GABA_A$ Rezeptor-Liganden beeinträchtigte das Vorkommen der internalisierten $GABA_A$ Rezeptoren jedoch nicht. Im Gegensatz dazu wurden AMPA Rezeptoren nach der Einwirkung von Agonisten rasch internalisiert und im Zellkörper akkumuliert. Die Existenz eines intrazellulären subsynaptischen Pools von $GABA_A$ Rezeptoren wurde auch unabhängig von Internalisierungsexperimenten nachgewiesen. Diese Resultate lassen vermuten, dass die Zirkulation von $GABA_A$ Rezeptoren zwischen der Zelloberfläche und dem subsynaptischen Pool einen Mechanismus zur Kurzzeit-Regulation der GABAergen synaptischen Übertragung darstellt. Vermutlich könnte dieser Regulationsmechanismus durch die chronische Behandlung mit Diazepam beeinflusst werden.

In der zweiten Studie wurde die Toleranz gegenüber der sedierenden Wirkung von Diazepam untersucht, wobei Mäuse mit einer Punktmutation in der α1-, α2-, α3- oder α5-Untereinheit während 8 Tagen mit dem Sedativum behandelt wurden. Der akut-sedative Effekt von Diazepam wird über die α1-$GABA_A$ Rezeptoren vermittelt. Wildtyp-, α2(H101R)- und α3(H101R)-Mäuse entwickelten Toleranz gegenüber der sedierenden Wirkung von Diazepam, was an einer verminderten Unterdrückung der motorischen Aktivität gezeigt werden konnte. Die α1(H101R) Mäuse wiesen keinerlei Veränderungen in der motorischen Aktivität auf, da die Punktmutation sie insensitiv gegenüber der Sedierung durch Diazepam macht. Die α5(H101R) Mäuse zeigten jedoch überhaupt keine sedative Toleranz nach chronischer Behandlung mit Diazepam. Daraus schliessen wir, dass die α5-$GABA_A$ Rezeptoren in die Toleranzentwicklung involviert sind. Autoradiographie mit [^{3}H]-Flumazenil in denselben Mäusen zeigte, dass die Gesamtmenge der $GABA_A$ Rezeptor Bindungsstellen nach chronischer Diazepam-Behandlung nicht reduziert war. Spezifische Liganden für die α5- $GABA_A$ Rezeptoren jedoch zeigten eine selektive Reduktion von Bindungsstellen im Gyrus Dentatus von toleranten Mäusen auf Wildtyp und α2(H101R), aber nicht α1(H101R). Diazepam-behandelte Wildtyp-Mäuse konnten in Kontrollexperimenten mit einer einmaligen Dosis Zolpidem sediert werden. Der Effekt dieses α1-$GABA_A$ Rezeptor-selektiven Liganden weist darauf hin, dass die α1-$GABA_A$ Rezeptoren auch nach der chronischen Diazepam-Behandlung immer noch funktionell sind. Des weitern fanden wir eine transiente Reduktion der motorischen Aktivität bei Wildtyp-Mäusen, die chronisch mit Zolpidem behandelt wurden, ohne dass die α5-Bindungsstellen reduziert worden wären. Zur Toleranzentwicklung aufgrund chronischer Diazepam-Anwendung, begleitet von einer Verminderung der α5- $GABA_A$ Rezeptoren im Gyrus Dentatus, müssen also α1- und α5-$GABA_A$ Rezeptoren miteinander aktiviert werden.

Zusammenfassend schliessen wir, dass sedative Toleranz gegenüber Diazepam durch die Beteiligung von bestimmten $GABA_A$ Rezeptor-Subtypen entsteht und nicht mit einem Funktionsverlust von α1- $GABA_A$ Rezeptoren assoziiert ist. Wahrscheinlich wird das Toleranzphänomen durch Anpassungsmechanismen in spezifischen neuronalen Kreisläufen hervorgerufen, die durch α1- und α5-$GABA_A$ Rezeptoren kontrolliert werden. Die konstitutive Endozytose stellt zwar einen wichtigen Mechanismus zur kurzzeitigen Regulierung der synaptischen Funktion von $GABA_A$ Rezeptoren dar, nimmt aber im Entstehen der Toleranz gegenüber Diazepam *in vivo* nur eine untergeordnete Rolle ein.

Summary

$GABA_A$ receptors ($GABA_AR$) mediate the major part of inhibitory neurotransmission in the brain. They are the target for several drugs such as the benzodiazepines, which are prescribed for their sedative, muscle-relaxant, anxiolytic and anti-epileptic properties. Tolerance to these properties develops after chronic use, but the mechanisms underlying the loss of drug effect remain unknown.

We used two independent approaches to investigate the mechanisms of $GABA_AR$ regulation. The first line of research involved the constitutive regulation of $GABA_AR$ in cell culture by endocytosis. The second approach concentrated on the mechanism of tolerance in mice carrying specific $GABA_AR$ subtypes made unresponsive to diazepam by a point mutation. The aim was to elucidate whether endocytosis is a possible mechanism for the development of tolerance. Furthermore, it is unknown if all major $GABA_AR$ subtypes contribute to the development of tolerance, a question that can be resolved by using mice with point-mutations in different $GABA_AR$ subtypes.

In the first study, an intracellular pool of $GABA_AR$ subunits was detected at presumptive postsynaptic sites. After tagging surface receptors of living hippocampal neurons with antibody and allowing for constitutive endocytosis, we found that surface receptors were distributed evenly over the membrane. Endocytosed receptors acccumulated in the pool underneath the synapse in a time-dependent manner, showing that the internalised receptors are relocated to the intracellular pool. The endocytosis occurred via clathrin-coated vesicles, as interfering with this pathway by cold buffer, hypertonic sucrose or a dynamin inhibiting peptide, greatly reduced the intensity of these internalised clusters. However, acute application of $GABA_AR$ ligands did not affect the appearance of the internalised $GABA_AR$. This is in contrast to AMPA receptors, which are rapidly internalized and accumulated within the cell body after agonist exposure. The intracellular $GABA_AR$ pool might provide a mechanism for short-term modulation of $GABA_AR$ and allow for rapid shuttling of receptors between the pool and the membrane. It is possible that this regulation of $GABA_AR$ is affected by chronic diazepam treatment.

In the second study, mice carrying a point mutation in either the α1-, α2-, α3- or α5-subunit were subjected to an 8-day treatment schedule with diazepam. Wild type, α2(H101R) and α3(H101R) mice developed tolerance to the sedative properties of diazepam, as demonstrated by the loss of motor depression after diazepam administration. The α1(H101R) mice did not display any sedation or tolerance, as the point-mutation renders them insensitive to sedation by benzodiazepines. The α5(H101R) mice however, did not show any sedative tolerance after chronic diazepam treatment, implying that α5-$GABA_AR$ subtypes are a prerequisite for the development of this tolerance. In addition, α5-$GABA_AR$ binding sites were only reduced in the dentate gyrus of tolerant wild type and α2(H101R) animals. This reduction was absent in α1(H101R) mice. Therefore specific

concurrent activation of both α1- and α5-$GABA_AR$ is required for this effect. We conclude that tolerance is the result of the simultaneously increased α1- and α5-mediated inhibition, which might lead to a reduction in α5-binding sites and activates adaptive mechanisms resulting in tolerance. This view was confirmed in wild type mice chronically treated with zolpidem, an α1-$GABA_AR$ selective ligand. These mice only displayed a partial motor depression after a similar treatment regimen, without any reduction in α5-binding sites. This partial tolerance appeared to be the result of a quicker recovery of the mice, as full sedation was observed directly after injection. Thus, only α1-mediated sedation is not enough to result in full sedative tolerance and the reduction in α5 binding sites. Moreover, diazepam-treated mice were sedated by a single dose of zolpidem, indicating that α1-$GABA_AR$ are still functional. This further implies that endocytosis of α1-$GABA_AR$ is not a major contributor to the development of sedative tolerance to diazepam.

In conclusion, endocytosis might be a mechanism involved in short-term regulation of $GABA_AR$, but it is not the main cause for the development of tolerance to diazepam *in vivo*. Sedative tolerance to diazepam requires activation of specific $GABA_AR$ and is not the effect of general enhanced inhibition through all $GABA_AR$ subtypes. It is likely caused by adaptive mechanisms specifically activated by concurrent enhanced GABAergic inhibition of both α1- and α5-$GABA_AR$.

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1 Introduction

γ -Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system. Its synaptic action is mediated mostly by $GABA_A$ receptors ($GABA_AR$), which belong to the superfamily of ligand-gated ion-channels. This receptor is a heteropentameric complex assembled from a family of at least 21 subunit genes (α1-6, β1-4, γ1-4, δ, ε, θ, π, ρ1-3). This heterogeneity results in a wide range of $GABA_A$ receptor subtypes in the brain, with a functional $GABA_A$ receptor typically consisting of 2α, 2β and 1γ subunit. The different α subunits have their own subtype-specific localisation in the brain, with the α1 subunit largely expressed in the cerebral cortex and the α2 subunit mainly in hippocampus and striatum (for detailed review: Fritschy and Brunig, 2003). The pharmacological properties of receptor subtypes are determined by their subunit composition. $GABA_A$ receptors containing an α1, α2, α3 or α5 subunit have a high affinity for classical benzodiazepines such as diazepam, whereas receptors containing an α4 or α6 subunit are diazepam-insensitive. Benzodiazepines are allosteric modulators of the $GABA_AR$ and they are widely prescribed for their sedative, anxiolytic, muscle-relaxant and anti-epileptic actions. Recent research has shown that different subtypes of $GABA_AR$ mediate benzodiazepine actions. In mice carrying an histidine to arginine point mutation in the α1-subunit (α1H101R), the diazepam binding to the α1 $GABA_AR$ was abolished, together with the sedative action of diazepam (Rudolph et al., 1999). Mice with a similar point-mutation in other α-subunits revealed that diazepam's anxiolytic effect is mediated by the α2-$GABA_AR$, and that the muscle-relaxant effect is partially regulated through α2- and α3-subunits (Löw et al., 2000). These findings open new prospects for therapy, with subtype-specific ligands devoid of side effects. Chronic treatment, however, often results in the development of tolerance, limiting the long-term use of benzodiazepines. The molecular mechanism of tolerance to benzodiazepines is as yet unknown. The subunit specificity for the actions of benzodiazepines raises the question whether the development of tolerance is mediated by specific $GABA_AR$ subtypes. A second possibility that has emerged is the view that tolerance to benzodiazepines stems from receptor internalisation (Tehrani and Barnes, 1997; Ali and Olsen, 2001). Research over the past years has revealed that synapses are not stationary connection points between neurons, but highly dynamic sites. The amount of receptors in the synaptic membrane is subjected to change, depending on the rates of synthesis, insertion, lateral mobility, endocytosis and subsequent degradation or recycling (Figure 1). This plasticity requires that postsynaptic components can be quickly inserted or removed from the postsynaptic density (PSD). The dynamic regulation of surface receptors is one mechanism

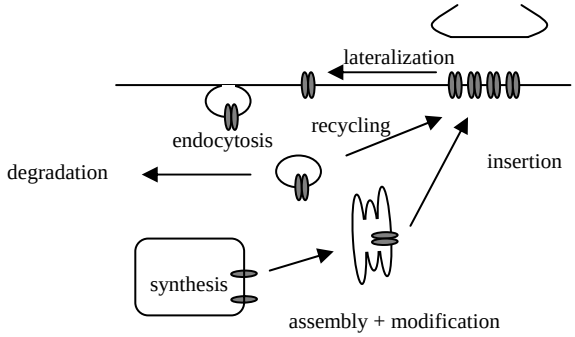

Fig 1.: Processes involved in regulation of membrane receptors

involved in phenomena such as long-term potentiation (LTP) and long-term depression (LTD).

There is evidence that various (chronic) drug treatments might not result in mRNA or protein expression changes, but may rather affect the receptor cycle mentioned above, influencing the (sub)cellular distribution of synaptic proteins and receptors (Tehrani and Barnes, 1997; Ali and Olsen, 2001; Kumar et al., 2003; review Thomas and Malenka, 2003), opening the possibility that tolerance is due to impaired receptor trafficking to the synapse. While relatively little is known about the molecular mechanisms of $GABA_AR$ trafficking, the regulation of AMPA receptors (AMPA R), which mediate excitatory neurotransmission, has already been extensively studied. The insertion and removal of AMPA R and $GABA_AR$ seem to be governed largely by the same protein kinases and protein phosphatases. Therefore the mechanisms involved in AMPA R regulation could give some insight into trafficking of the $GABA_A$ R. Below I will summarise current knowledge on trafficking, membrane stability and endocytosis of both receptor types and, with regard to the problem of benzodiazepine tolerance, the effects of (chronic) drug exposure on their surface expression will be compared.

1.1 AMPA receptors

The postsynaptic density (PSD) of glutamatergic synapses contains a large diversity of neurotransmitter receptors, signalling molecules and scaffolding proteins (review Kennedy, 1997, see also Fig.2 on page 8). It has one or more of the following glutamate receptors: NMDA R, AMPA R and kainate receptors. The central component of the glutamatergic PSD is the scaffolding protein PSD-95 through which the glutamate receptors are clustered at the synapse. This protein has several PDZ (**P**SD-95, **D**lg, **Z**O-1- homology) domains, which are modular protein domains that can interact with C-terminal domains of other proteins. It is believed that perhaps several hundreds of proteins are brought in close vicinity of each other in the PSD through interactions with PDZ-domains of PSD-95 and

other proteins (Sheng, 2001; Sheng and Kim, 2002). AMPA R are linked to PSD-95 via a family of transmembrane AMPA R regulatory proteins (TARPs), among them a protein called stargazin (Bredt and Nicoll, 2003; Tomita et al., 2004).

AMPA R are glutamate-gated channels that mediate a large part of excitatory transmission in the brain. Upon glutamate binding, the channel opens, allowing Na^+ influx into the neuron. AMPA R consist of a combination of the subunits GluR1-4, possibly in a tetrameric conformation. The permeability of the receptor for calcium is determined by the presence of a glutamine in transmembrane region 2. The GluR2 subunit contains an arginine instead of glutamine, making the receptor impermeable for Ca^{2+}. Through RNA editing, a process in which adenosine is converted into inosine, the glutamine codon (CAG) is changed into an arginine codon (CGG) (Sommer et al., 1991). As *in vivo* the prevalent heteromers are either build up from GluR1/2 or GluR2/3 subunits, the majority of AMPA receptors is Ca^{2+} impermeable. The GluR4 subunit is only expressed during development and can replace the GluR1 subunit in GluR1/2 heteromers. The GluR1 and the GluR4 subunits have a long cytoplasmic C-tail, whereas GluR2 and GluR3 subunits have a short one. This intracellular tail contains several sequences for interaction with proteins involved in trafficking, anchoring or phosphorylation of AMPA R. The longer C-tail of GluR1 and GluR4 harbours extra domains, leading to differences in transport, insertion and removal from the membrane compared to the GluR2 and GluR3 subunits (Shi et al., 2001). GluR2/3 heteromers are responsible for basal synaptic transmission in the brain, which is severely reduced in GluR2/3 double knockout mice (Meng et al., 2003). On the other hand, AMPA R heteromers with a GluR1 subunit are incorporated in the synapse in response to synaptic activity leading to long-term potentiation (LTP) (Hayashi et al., 2000), which is absent in GluR1 knockout mice (Zamanillo et al., 1999). In order to understand the regulation of AMPA R, the interactions of the GluR1 and the GluR2 subunit with trafficking and stabilising proteins in the postsynaptic density have been studied extensively.

1.1.1 Exocytosis & synaptic stability of AMPA receptors

- GluR2 subunit

GluR2/3 AMPA R heteromers cycle constitutively in and out of the synapse, evidence that they must be internalised and inserted constantly (Ehlers, 2000; Lin et al., 2000). Three of the proteins involved in exocytosis and synaptic stability will be highlighted below. One of the first proteins shown to bind to GluR2 was NSF (N-ethylmaleimide Sensitive Factor) (Nishimune et al., 1998), an ATP-ase known at that time to be involved in presynaptic exocytosis. Infusing a peptide that blocks the GluR2-NSF binding resulted in decreased surface expression (Noel et al., 1999) through increased endocytosis (Braithwaite et al., 2002). Intriguingly, the NSF binding site partially overlaps with that of AP2, an adaptor

protein involved in clathrin-coated vesicle endocytosis (Lee et al., 2002). Loss of NSF-binding would therefore free the GluR2 receptor for AP2 binding and consequent endocytosis. Binding to NSF apparently brings AMPA R containing the GluR2 subunit to the membrane and prevents their endocytosis.

Another part of the intracellular domain of GluR2 interacts with GRIP/ABP protein (glutamate receptor interacting protein/ AMPA R binding protein). Different hypotheses circulate concerning the role of GRIP/ABP in AMPA R regulation. When the interaction with GRIP/ABP was disrupted by mutation of the GluR2 cytoplasmic tail, stable incorporation of this subunit into the membrane of cultured hippocampal neurons was prevented (Seidenman et al., 2003), review (Barry and Ziff, 2002). These imply that GRIP/ABP is involved in synaptic stabilisation of GluR2. This is in contrast with other reports that abolishing GRIP/ABP-GluR2 did not affect surface levels, only the amount of GluR2 that remained internalised after endocytosis (Braithwaite et al., 2002), (Fu et al., 2003), suggesting that it interacts with the intracellular pool of GluR2. Since this PDZ protein is found in excitatory synaptic sites and in intracellular compartments (Burette et al., 2001) both hypotheses could be correct. A third interacting PDZ protein is PICK-1 (Protein that Interacts with Kinase C), which, like PSD-95, is involved in clustering of AMPA R (Xia et al., 1999). By binding PKC, PICK-1 might also cluster PKC at synaptic sites where it can phosphorylate AMPA R, thus influencing their surface stability (see section on phosphorylation).

- GluR1

In contrast to the constitutive cycling of GluR2/3 heteromers, the longer C-tail retains the GluR1 subunits intracellularly and their insertion in the plasma membrane is activity-dependent. In hippocampal cell cultures and slices, neuronal activity causes an influx of Ca^{2+} that consequently results in coactivation of CamKII (Shi et al., 2001) and PKA, which in turn phosphorylates GluR1 in the Golgi (Ehlers, 2000; Esteban et al., 2003). The phosphorylation of the C-tail releases the retention and the GluR1 subunits are inserted into the synapse. The GluR4 subunit is more expressed than GluR1 in early development, and this subunit does not require the coactivation of CamKII for synaptic incorporation. The trafficking of GluR1 to the membrane depends also on interaction of the GluR1 subunit with the PDZ-protein SAP97, which binds to GluR1 subunits in the ER and Golgi. At the synapse, however, only a few AMPA R are found connected to this protein (Sans et al., 2001). Mutation of the SAP97 binding site disrupts synaptic accumulation of GluR1 subunits, another argument that SAP97 is important for delivery of the receptors to the synapse (Leonard et al., 1998). It must be stated that, although not so many interacting proteins are known for GluR1 as for GluR2, GluR1 is usually expressed as a GluR1/2 heteromers and is therefore indirectly linked to the stabilising proteins that bind to GluR2.

1.1.2 Endocytosis of AMPA receptors

- Phosphorylation

Endocytosis of AMPA R is well studied and several components influencing the rate of internalisation are known. One important signal that determines whether a protein stays or is removed from the surface is its phosphorylation state. Phosphorylation can enhance or disrupt binding to interacting proteins and thereby affect cell surface stability and trafficking. AMPA R contain several consensus sites for phosphorylation by different protein kinases (See Table on page 17). In particular, the C-tail of the GluR1 subunit harbours phosphorylation sites for PKA and CamKII, whereas the GluR2 subunit can be phosphorylated by PKC. Synaptic incorporation of GluR1 involves phosphorylation at Ser845, which can be induced by agonist stimulation as mentioned previously; its removal by endocytosis is associated with dephosphorylation by the protein phosphatase calcineurin at this site (Ehlers, 2000; Esteban et al., 2003; Snyder et al., 2003). Also, agonist stimulation is known to redistribute GluR1 away from the synapse (Lin et al., 2000). Whether this effect results from-, or causes dephosphorylation of GluR1 is not yet clear, although recent evidence points to the latter: It has been shown that the GluR2 subunit is very mobile; it can move laterally within the membrane and is more stationary at synapses than extrasynaptically (Borgdorff and Choquet, 2002). As GluR2 is expressed at the cell-surface as a heteromer, GluR1/2 or GluR2/3 heteromers can move out of the synapse and can be internalised perisynaptically, where hotspots of clathrin, a component of endocytic vesicles, are found (Blanpied et al., 2002). Furthermore, the protein phosphatase calcineurin can associate to these clathrin-coated vesicles via ampiphysin, a protein involved in clathrin-coated vesicle endocytosis. The calcium influx resulting from AMPA or NMDA receptor stimulation activates calcineurin. By its interaction with ampiphysin, calcineurin can possibly alter endocytosis rates (Lai et al., 1999; Beattie et al., 2000) and its close proximity to endocytosed GluR1 could lead to the dephosphorylation of Ser845 (Ehlers, 2000; Snyder et al., 2003).

For the GluR2 subunit, the effect of phosphorylation appears to be opposed to that of the GluR1 subunit; Phosphorylation at Ser880 at the cytoplasmic tail of GluR2 reduces the binding affinity of this subunit for GRIP/ABP, resulting in subsequent endocytosis (Matsuda et al., 2000; Seidenman et al., 2003). Binding of PICK-1 is not affected and it is believed that a second function of PICK-1 might be to prime the receptors for endocytosis (Chung et al., 2000; Seidenman et al., 2003).

On the other hand, a recent study has shown that it might not be the phosphorylation that induces the endocytosis of GluR2, but the glutamate binding itself (Tomita et al., 2004). These authors demonstrated that binding of glutamate results in a conformational change of

the receptor that releases its connection with stargazin, freeing the AMPA R for endocytosis.
The stimulus that leads to endocytosis also determines which endocytic pathway the receptor enters. Receptors that are endocytosed upon NMDA or AMPA stimulation enter a recycling endosome pathway, whereas insulin stimulation activates a calcium-independent pathway that results in the accumulation of receptors in an unknown, non-lysosomal, compartment (Beattie et al., 2000; Lin et al., 2000).

- Ubiquitination

Ubiquitination targets proteins to the proteasome, where they can be degraded (for review Hicke, 2001). Recent research points to a role of ubiquitination in AMPA receptor regulation. Blocking ubiquitination prevented agonist-induced (AMPA) endocytosis of GluR1 and GluR2 (Patrick et al., 2003). Whether ubiquitination of AMPA R plays a role in their endocytosis has not been investigated. However, another study demonstrated that ubiquitination of PSD-95 leads to reduction of PSD95 at synapses due to degradation by the proteasome and consequent reduction of AMPA R (Colledge et al., 2003). With fewer PSD-95 available, fewer AMPA R can be clustered at the synapse, leading to reduced AMPA currents.

1.1.3 AMPA receptor regulation in LTP and LTD

The molecular cues and signals mentioned above come into play in AMPA R regulation and have been studied mainly in response to acute stimuli. As mentioned before, synaptic activity is needed for GluR1 containing AMPA R to be inserted in the membrane. In so-called silent synapses (synapses that contain only NMDA receptors), NMDA R stimulation leads to *de novo* incorporation of GluR1/2 heteromers and 'unsilencing' of the synapse. In synapses that contain GluR2/3 heteromers, activation by either AMPA or NMDA results in a replacement of GluR2/3 heteromers by GluR1/2-containing receptors. Thus, the regulating subunit for constitutive cycling is the GluR2, and the dominant subunit for activity-dependent insertion is GluR1 (Shi et al., 2001).
AMPA R regulation is also studied extensively in hippocampal slices, as stimuli leading to the induction of long-term potentiation (LTP) or long-term depression (LTD) have a profound effect on the surface levels and the endo/exocytosis rates of AMPA R. LTP and LTD are considered as a molecular substrate of learning. For LTP, in short, a high frequency stimulation protocol leads to an increased synaptic strength that lasts for hours. For LTD, low frequency stimulation results in a decreased synaptic strength. Most of the studies described below investigated LTP and LTD in the CA1 region of the hippocampus, where both LTP and LTD are NMDA R dependent (Liu et al., 2004). The increase in

synaptic response seen with hippocampal LTP involves the GluR1 subunit: LTP is intact in GluR2/3 knockout mice (Meng et al., 2003) and is absent in GluR1 knockouts (Zamanillo et al., 1999). In the CA1 of the hippocampus, LTP is induced upon NMDA-R stimulation, which in turn activates CamKII that phosphorylates the GluR1 subunit at Ser 831 (Mammen et al., 1997). The effect on GluR1 seems to involve 2 mechanisms (review: Bredt and Nicoll, 2003): the first one involves a direct phosphorylation of AMPA R by CamKII, thereby facilitating the synaptic incorporation of this AMPA R. At the same time, GluR2/3 heteromers are removed from the synapse, as phosphorylation by PKC leads to their endocytosis. The netto result is the replacement of GluR2/3 by GluR1/2 heteromers. There is a second mechanism necessary for the increased membrane expression of GluR1, since mutation of the Ser831 did not prevent the synaptic incorporation of GluR1 subunits. Possibly, an unknown protein is phosphorylated by CamKII and brings the AMPA R to the surface (Hayashi et al., 2000). One likely candidate would be SAP-97.

While GluR1 incorporation is necessary for LTP, GluR2 endocytosis is essential for the induction of LTD. Transfecting cerebellar granule cell cultures that lacked GluR2 with a point mutated GluR2 subunit, which mimicked phosphorylation at Ser880, restored LTD (Chung et al., 2003). Thus, GluR2 phosphorylation and its subsequent internalisation are required for LTD induction. LTD not only involves increased GluR2 endocytosis, but PDZ proteins (e.g. GRIP/ABP) also bind to these internalised receptors and prevent their reinsertion in the plasma membrane (Daw et al., 2000). In conclusion, synaptic activity can influence interaction of AMPA R with trafficking or stabilising proteins by changing the phosphorylation state of the subunit. The differences on the molecular level between GluR1 and GluR2 containing AMPA R, seen as a difference in the length of the C-tail, are reflected in the function that the different AMPA heteromers have, with GluR2/3 heteromers responsible for basal transmission and GluR1/2 heteromers for LTP.

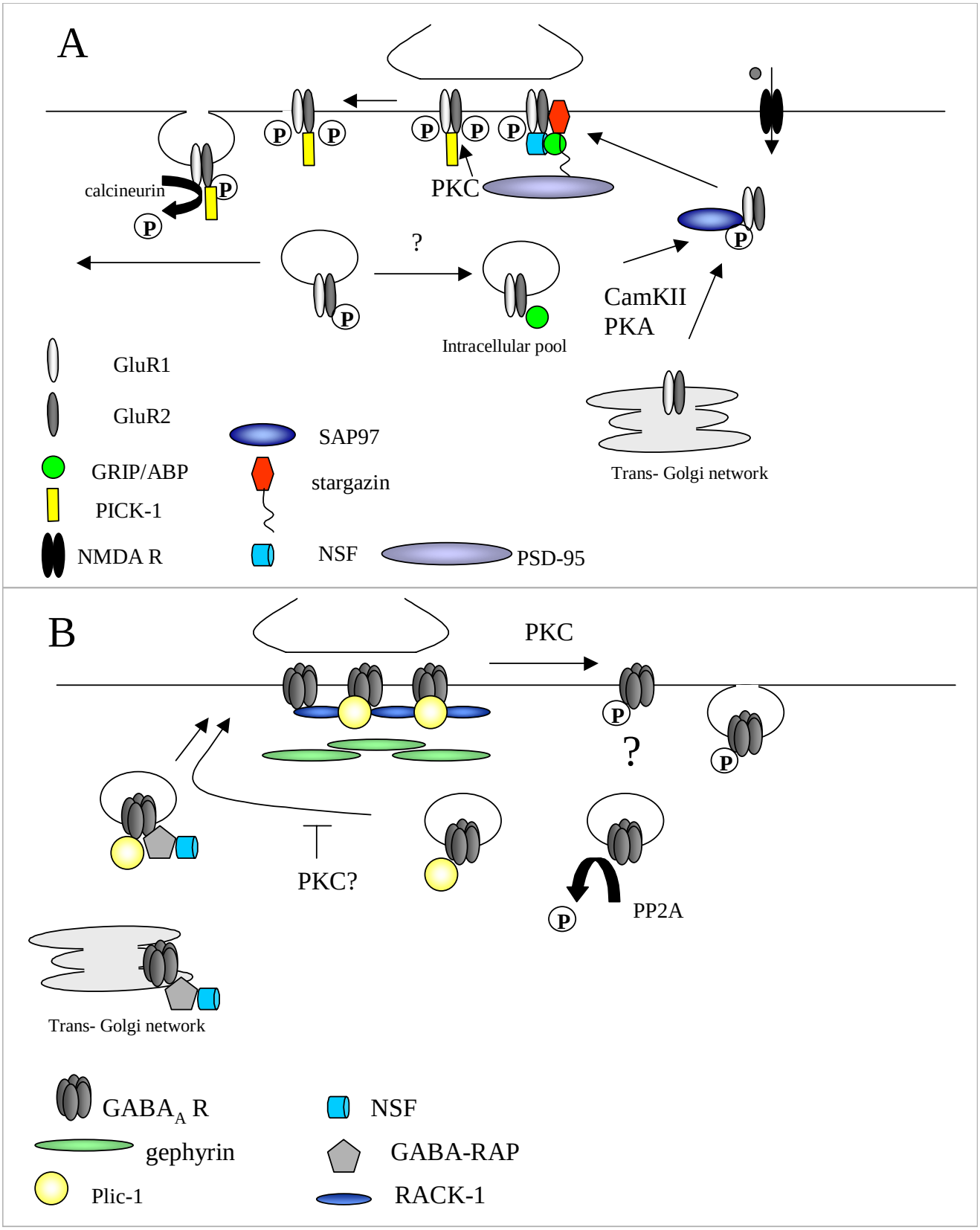

Fig. 2 : Regulation of AMPA receptor (A) and GABA$_A$ receptors (B) at the synapse by (de)phosphorylation.

1.2 $GABA_A$ receptors

$GABA_AR$ are ligand-gated ion channels mediating inhibitory neurotransmission in the brain. Upon GABA binding, the channel opens and the resulting chloride influx will cause a hyperpolarization and an increase in conductance, making it more difficult for the neuron to reach threshold for eliciting an action potential. The PSD of GABAergic synapses contain, in contrast to the glutamatergic PSD, far fewer identified proteins. In electron microscopy, the PSD of GABAergic synapses appears thinner and less electron dense than that of glutamatergic synapses. Studies in brain tissue and primary neuronal cultures have shown that $GABA_A$ R are localized postsynaptically, as they colocalize with gephyrin, a marker for the GABAergic postsynaptic density (Rao et al., 2000; Brunig et al., 2002a). $GABA_AR$ are synthesised in the ER and assembled into heteromers in the Golgi apparatus (Moss and Smart, 2001). In contrast to AMPA R, it is unclear what the exact molecular signals are that lead to the translocation of $GABA_AR$ from the Golgi to the postsynaptic density. It is probably independent of activity, since blocking synaptic activity has no effect on postsynaptic clustering of $GABA_AR$ (Craig et al., 1994; Studler et al., 2002). Nevertheless, some proteins that are involved the trafficking to the PSD and in the postsynaptic stability and clustering of $GABA_AR$ have been identified (see also Fig. 2).

1.2.1 Synaptic incorporation and stabilisation of $GABA_A$ receptors

Using a yeast-two-hybrid screen with the intracellular loop of the γ2 subunit as bait, a 17-kD protein has been identified that specifically interacts with the γ2-$GABA_AR$ subunit (for review: Kneussel, 2002). Though this $GABA_AR$ associated protein (GABARAP) can bind to the γ2 subunit, gephyrin and microtubules, it is not enriched at the synapse. Rather, GABARAP is found in intracellular compartments, such as the Golgi apparatus, and is most likely involved in trafficking the $GABA_AR$ to the synapse. The protein NSF, which is responsible for AMPA R stability at the synapse, can bind to GABA-RAP and helps bringing the vesicles with $GABA_AR$ to the membrane (Kneussel, 2002). Where exactly the $GABA_AR$ are inserted in the membrane remains to be elucidated. At the synapse, $GABA_AR$ form clusters that colocalize with gephyrin. This is thought to be a scaffolding molecule, as it can interact with itself and with microtubules and can thus create a lattice for proteins at the synapse. In contrast to PSD-95 in the glutamatergic synapse, gephyrin lacks PDZ-domains. Although direct binding of gephyrin with the β subunit of the glycine receptor has been shown to trap and accumulate glycine receptors either intracellularly or at the membrane (Kirsch et al., 1995; Meier et al., 2001; Hanus et al., 2004), no direct interaction between gephyrin with $GABA_A$ R has ever been demonstrated (Kannenberg et al., 1997; Meyer et al., 2000). A functional interdependence of gephyrin and $GABA_AR$ has been

shown, though, in studies using knockout mice. Postsynaptic clustering of GABA R and gephyrin is impaired in $\gamma_2^{-/-}$ mice, showing the role of the γ2 subunit in formation of these clusters. Gephyrin clusters are absent as well, illustrating that gephyrin needs the $GABA_AR$ to go to the synapse (Essrich et al., 1998). Abolishing γ2 subunit expression in the forebrain after brain maturation using Cre-CamKII mediated recombination resulted in a reduction of GABA R and gephyrin clusters, as well (Schweizer et al., 2003). This suggests that the γ2 subunit is not only necessary for initiation of receptor clustering, but is also needed for maintaining these clusters. With fewer clustered receptors present, the gephyrin is also removed from the synapse, pointing towards a dynamic regulation of gephyrin. Results from gephyrin$^{-/-}$ mice are less conclusive: In one study it was demonstrated that $GABA_A$ receptor clustering is largely absent in gephyrin$^{-/-}$ mice (Kneussel et al., 1999). However, a recent report showed that $GABA_AR$ clusters are present in cultured gephyrin$^{-/-}$ hippocampal neurons (Levi et al., 2004). This could reflect improvement of culture conditions, in which $GABA_AR$ can cluster at postsynaptic sites in a gephyrin-independent way.

Another protein found clustered at the synapse is dystrophin, a member of the dystrophin-associated protein complex (DPC). In *mdx* mice, which lack dystrophin, a strong reduction of $GABA_AR$ receptor clusters was detected, although gephyrin clustering was unaffected, showing that it is able to stabilise $GABA_AR$ at the synapse (Knuesel et al., 1999). However, in dystroglycan-deficient neurons that lacked dystrophin immunoreactivity, $GABA_AR$ clustering was intact (Levi et al., 2002). A hypothesis is that the DPC could provide a scaffold at the PSD, enabling changes in postsynaptic $GABA_AR$ number without loosing the postsynaptic apparatus (Fritschy and Brunig, 2003). Whether other proteins of the DPC are involved in $GABA_AR$ clustering still remains to be investigated.

A further, recently discovered $GABA_AR$ interacting protein, Plic-1, is an ubiquitin-like protein that can bind to α and β subunit isoforms and is localised in both synapses and intracellular compartments. By binding to the $GABA_AR$ Plic-1 prevents degradation of $GABA_AR$ by the proteasome. Half-life times and number of surface $GABA_AR$ increased when Plic-1 was coexpressed with $GABA_AR$ in HEK293 cells. It is thus thought that Plic-1 facilitates insertion of $GABA_AR$ in the membrane through stabilising the intracellular pool by preventing their degradation after endocytosis (Bedford et al., 2001).

1.2.2 Endocytosis of $GABA_AR$

- Phosphorylation

It is believed that phosphorylation of $GABA_AR$ subunits can influence surface receptor numbers in a similar manner as seen with AMPA R (review Kittler and Moss, 2003). Several phosphorylation sites were identified on $GABA_AR$ subunits that are targets for PKA and PKC (see table on page 17). The Ser 409 site of the β1 and Ser 408/409 of the β3

subunit are a major substrate for PKA, whereas β2 does not contain any PKA-sites. PKA is targeted to the $GABA_AR$ by A-Kinase Anchoring Proteins (AKAPs). AKAP is a PKA adaptor protein that selectively interacts with β1 and β3 but not β2 subunits (Brandon et al., 2003).
PKC can phosphorylate the β- and γ-subunits (Ser 409 for β1, Ser410 for β2, Ser 408/409 for β3 and at Tyr365/367 for γ2), but not α subunits (Krishek et al., 1994; Connolly et al., 1999; Brandon et al., 2002a). PKC is directly associated to the β1 subunit intracellular loop from residue 405-415 and can then phosphorylate the corresponding residues. This is enhanced by the Receptor for Activated C-kinase (RACK-1), which binds to $GABA_AR$ β1 subunits directly upstream of PKC. Although RACK-1 is not necessary for PKC binding to $GABA_A$ R, it does increase the amount of phosphorylation at Ser409 (Brandon et al., 2002b). Inhibiting RACK-binding also decreases functional modulation by muscarinic M1 G-protein coupled receptors that activate PKC. Thus it seems that RACK-1 has a comparable role as PICK-1 for AMPA R in controlling the amount of phosphorylation of $GABA_AR$.

Although progress has been made in identifying $GABA_AR$ substrates for protein kinases, how they affect receptor surface numbers of the $GABA_AR$ is not clear-cut. The effects of PKA depend critically on the phosphorylated residues and subunits: Phosphorylation of Ser 409 of β1 in α1β1γ2 recombinant receptors resulted in a decreased GABA-response, whereas phosphorylation of both Ser408 and 409 in β3-containing receptors lead to potentiation of the GABA response (McDonald et al., 1998). Mutating Ser408 into an alanine in the β3-subunit converted the potentiation in an inhibition, mimicking the results seen with β1-subunits. In another study, inhibition of PKA decreased surface α1β2γ2-$GABA_AR$ levels in transfected Sf9 cells (Ali and Olsen, 2001). This is interesting, since none of these subunits contains any PKA-phosphorylation sites, implying that another protein must play a role in this mechanism. In recombinant systems, phosphorylation of β2/3 by PKC (Krishek et al., 1994; Filippova et al., 2000; Brandon et al., 2002a) resulted in reduced GABA currents due to increased endocytosis of the $GABA_AR$ subunits. In neurons, the β3-subunit is constitutively phosphorylated at Ser408/409 and this could therefore be important for constitutive regulation of receptors in the synaptic membrane. In superior cervical ganglion neuron cultures, PKC activation resulted in increased endocytosis of β1 containing $GABA_AR$ (Brandon et al., 2002b), which is similar to the effect of PKA on β1 subunits mentioned above.
Whether phosphorylation of the $GABA_AR$ always results in significant endocytosis in neurons is unclear: Evidence from α1β2γ2 transfected HEK293 cells suggests that PKC does not affect endocytosis itself, but blocks receptors from returning to the surface. This downregulation of receptors from the surface seems to be independent from PKC

phosphorylation of the γ subunit, as mutation of these PKC sites had no effect (Connolly et al., 1999). Data from studies with PKA also suggest that $GABA_AR$ dynamics mediated by PKA are also not necessarily regulated through phosphorylation of the receptor itself (Ali and Olsen, 2001; Lilly et al., 2003). Perhaps another protein that is involved in surface stability is affected by PKA and PKC, resulting in loss of surface receptors. Another hypothesis is that phosphorylation influences channel desensitization rather than surface localization (Hinkle and Macdonald, 2003; Jovanovic et al., 2004), as PKC activation did not change receptor surface numbers in cultured cortical neurons (Brandon et al., 2000). More research is therefore needed to elucidate the exact role that phosphorylation plays in $GABA_AR$ dynamics in neurons.

1.2.3 Dynamic regulation of $GABA_AR$

Endocytosis of $GABA_AR$ has been studied in response to BDNF. BDNF is a growth factor that can act via the TrkB or p75 receptor. Application of BDNF on hippocampal neurons lead to a reduction of mIPSCs within minutes and this effect lasted for several hours (Brunig et al., 2001). This decrease was accompanied by a diminution of GABAR surface labelling. In contrast, Jovanovic et al (Jovanovic et al., 2004) showed a biphasic effect of BDNF on mIPSCs: an increase was detected in the first minutes, followed by a decrease as seen by Brünig et al. The biphasic effect was mirrored in the phosphorylation state of the β3-subunit: phosphorylation directly after BDNF exposure was mediated by PKC /RACK, whereas PP2A was responsible for dephosphorylation at the time when the decrease in mIPSCs was detected. However, no biphasic effect was seen on $GABA_AR$ surface levels.
In cortical neurons the phosphorylation of β3-subunits of $GABA_AR$ and the associated reduction of GABA currents can be induced through muscarinic achetylcholine receptors via a G-protein coupled mechanism that activates PKC (Brandon et al., 2000). Furthermore, RACK-1 is implicated in the PKC-dependent regulation via $5\text{-}HT_2$ R (Feng et al., 2001). Dopamine receptors (DR) can play a role as well. Dopamine agonists reduced GABA currents in neostriatal neurons (Flores-Hernandez et al., 2000) and D4R in prefrontal cortex inhibited $GABA_AR$ function (Wang et al., 2002). First indications are that dopamine receptors do this via a PKA/PP1 signalling pathway for both AMPA R and $GABA_A$ R (Wang et al., 2002; Wolf et al., 2003).

The regulation of $GABA_AR$ through phosphorylation appears more complex than that of AMPA R. The opposite effect of phosphorylation on the GABA response with the β1 or the β3 subunits in recombinant systems leaves room for differential modulation of GABAergic transmission in several brain regions depending on the expression patterns of these subunits.

1.3 Regulation of cell surface receptors by chronic drug use

Synaptic activity can directly or indirectly regulate the amount of cell surface receptors in glutamatergic and GABAergic synapses. Acute and chronic administration of approved drugs or substances of abuse influences the synaptic activity in specific brain areas depending on their mode of actions. It is possible that changes in transmission will affect consequently the amount of receptors present in the postsynaptic membrane. Some of the effects that drugs have on AMPA R and $GABA_A$ receptor regulation will be discussed below.

1.3.1 AMPA receptors

Although AMPA R do not represent a pharmacological target in clinical use, chronic administration of drugs of abuse is associated with changes in AMPA receptor-mediated signalling and/or synaptic strength. Although most studies on LTP/LTD have focussed on the hippocampus or cerebellum, similar forms of potentiation also exist in the mesolimbic system, which plays an important role in the development of drug abuse and craving. Considerable evidence indicates that AMPA R mediate major down-stream effects of drugs activating the mesolimbic system. In the midbrain (ventral tegmental area) a single dose of cocaine resulted in increased synaptic strength due to an upregulation of number/function of AMPA receptors (Ungless et al., 2001). This seems to be true for several drugs of abuse with different mechanisms of action (review Thomas and Malenka, 2003). Another study demonstrated in nucleus accumbens cultures that stimulation of D1 receptors led to enhanced phosphorylation of GluR1 at the PKA site. This effect increased the GluR1 surface expression levels accordingly, by changing the rate of externalisation (Chao et al., 2002; Wolf et al., 2003). Moreover, local overexpression of GluR1 via injection of herpes simplex viral vector in the rostral VTA can enhance stimulant and rewarding properties of morphine (Carlezon et al., 1997). It is intriguing to speculate that drugs of abuse might induce drug addiction by enhancing synaptic strength of glutamatergic synapses in the reward pathway via a dopamine-dependent mechanism.

Other drugs also have effects on AMPA surface levels. Chronically treated hippocampal cultures with the antimanic agents lithium and valproate had reduced surface expression of GluR1, and synaptosomes of chronically treated rats also expressed less GluR1 (Du et al., 2003). In contrast, the antidepressants desipramine and paroxetine, selective noradrenaline and serotonin uptake inhibitors respectively, resulted in increased GluR1 and GluR2/3 in membranes of hippocampus, but not of cerebral cortex (Martinez-Turrillas et al., 2002). Several drugs might exert their actions by indirectly influencing glutamatergic synapses,

most likely through crosssignalling via PKA, PKC, or CamKII. This might provide a way for the development of adaptive mechanisms upon chronic drug treatment.

1.3.2 $GABA_A$ receptors

As mentioned in the beginning, a major class of drugs acting on the $GABA_AR$ is the benzodiazepines. They are positive allosteric modulators of GABA, thereby increasing the synaptic inhibition in the CNS. Tolerance to benzodiazepines develops readily after chronic use. Although the mechanism of tolerance is not known, the degree of tolerance and the time scale in which it is induced depends on the dose, the intrinsic activity of the ligand used, as well as its selectivity for certain $GABA_AR$ subtypes (Bateson, 2002). Tolerance to diazepam is for instance more readily induced when several low doses are given per day, thus keeping constant $GABA_AR$ occupation levels, then with a single high dose per day (Hutchinson et al., 1996b). Furthermore, ligands like zolpidem that are selective for the α1-$GABA_AR$ have less tolerance liability than ligands that bind to all diazepam-sensitive receptors (Rush, 1998). Tolerance is not associated with major changes in $GABA_AR$ subunit expression and, in several experimental paradigms, is readily reversible with a single dose of the benzodiazepine antagonist flumazenil (Gonsalves and Gallager, 1988; Tietz et al., 1999b). One of the possible causes of tolerance has been suggested to be the 'uncoupling' of the GABA-binding site and the benzodiazepine-binding site on the receptor. The lack of diazepam effect originates from the loss of the interaction between these two sites. Alternatively, a recent report suggested that tolerance to benzodiazepines might be due to induction of endocytosis of $GABA_AR$. In transfected sf9 cells the number of $\alpha1\beta2\gamma2$ $GABA_AR$ in the membrane decreased after chronic diazepam exposure and the main part of the receptors was detectable intracellularly. Another study demonstrated that after 7 days of lorazepam treatment, [^{3}H]flunitrazepam-binding and α1 protein levels were increased in clathrin-coated vesicles and decreased in synaptosomes of mice (Tehrani and Barnes, 1997). The observation that receptors can be internalised after chronic diazepam exposure is consistent with the uncoupling-hypothesis. Diazepam is cell-permeable and can bind to internalised $GABA_AR$ whereas GABA cannot. Subsynaptic internal receptors might be quickly recruited back to the membrane surface after flumazenil exposure, resulting in 'recoupling' since GABA and diazepam can now bind to the same receptor again. Internalisation of $GABA_A$ R after benzodiazepine exposure would also explain why clear changes in $GABA_AR$ expression and binding sites were not observed in several studies in tolerant animals (review Hutchinson et al., 1996a). Only long-term treatment with high doses of benzodiazepines seems to result in changes in expression levels. A recent model of tolerance states the following: initial potentiation of the GABA response is followed by desensitisation. This can be a cue for endocytosis, which results in the uncoupling of the GABA- and benzodiazepine site. In the long run, the endocytosed receptors could influence gene transcription, as seen in chronically treated animals (Bateson, 2002). The mechanism

of benzodiazepine-induced endocytosis is not resolved yet, although results from a heterologous expression system suggest that PKA inhibition might play a role (Ali and Olsen, 2001). In another study, a one-week flurazepam treatment in rats, known to result in anti-convulsant tolerance, decreased PKA activity and mIPSC amplitude in CA1 pyramidal cells (Lilly et al., 2003). How benzodiazepine exposure affects PKA activity remains to be investigated. Major unresolved issues, however, are whether all major $GABA_AR$ subtypes contribute to the development of tolerance to benzodiazepines, and whether the mechanisms of tolerance are the same for all benzodiazepine site ligands. In addition to benzodiazepines, chronic alcohol administration can induce internalisation of α1-$GABA_AR$ subtypes in rat cerebral cortex (Kumar et al., 2003). This effect was subunit specific and might be under the control of PKC, as the association of PKCγ with α1-$GABA_AR$ was reduced after chronic ethanol exposure (Kumar et al., 2002).

1.3 Comparison of AMPA receptors versus $GABA_A$ receptors

It is interesting to note that several treatment paradigms lead to opposite effects when comparing AMPA R with $GABA_AR$ (see table). First, protocols that induce LTP in glutamatergic synapses decrease the function of GABAergic synapses. NMDA R-dependent LTP of glutamatergic synapses in the CA1 region of the adult hippocampus involves a rise in calcium through the NMDA R, leading to LTD in GABAergic synapses (Stelzer and Shi, 1994; review Gaiarsa et al., 2002). This LTD is accounted for by a reduction in $GABA_AR$ efficacy and is calcineurin dependent (Lu et al., 2000). Similarly, dendritic $GABA_AR$ activity in cerebellar granule cells is partially blocked by NMDA R via calcineurin dephosphorylation (Cupello and Robello, 2000). It is not clear, however, which $GABA_AR$ subunits calcineurin dephosphorylates and how this may lead to LTD.
Insulin stimulates endocytosis of AMPA R (Beattie et al., 2000; Lin et al., 2000; Man et al., 2003), whereas it increases β2 subunit-dependent exocytosis of $GABA_AR$ and enhances the amplitude of mIPSCs in HEK293 cells and cultured hippocampal neurons (Wan et al., 1997). Even dopamine receptor stimulation produces different effects on the two receptor types, although studies were carried out in different types of preparations: application of a D1 agonist lead to increased GluR1 surface levels trough a PKA-dependent mechanism in nucleus accumbens cultures (Chao et al., 2002), whereas GABA currents were reduced in neostriatal neurons via a PKA/DARPP-32/PP1 pathway (Flores-Hernandez et al., 2000). In general it can be said that both receptor types are regulated by similar signalling pathways, but with different end results. An open question is if these signalling pathways affect one or more synapse within the same cell, and how many synapses could be modulated at the same time.

The glutamatergic system is also affected by chronic diazepam use. Increases in NMDA R and AMPA R levels were reported after chronic benzodiazepine treatment and during the withdrawal phase (Tsuda et al., 1998; Izzo et al., 2001), and glutamate antagonist were able to prevent diazepam dependence (Steppuhn and Turski, 1993). The changes in these studies are related to the development of dependence and the withdrawal symptoms that occur after cessation of the treatment. However, it is conceivable that the glutamatergic system can contribute to the development of tolerance, as NMDA R subunit mRNA is upregulated after 4 days of benzodiazepine exposure (Perez et al., 2003) All these observations imply that it will be essential in the future to investigate if or how inhibitory and excitatory synapses 'interact' to changes induced by either stimuli or drugs.

Conclusions

The regulation of AMPA R and GABA R is very different: where AMPA R are directly influenced by synaptic activity, $GABA_AR$ are modulated through other types of receptors. We summarized here the most important of the many proteins and factors known to play a role in AMPA R trafficking. On the other hand, information on $GABA_AR$ regulation is only slowly emerging. $GABA_AR$ are a target for benzodiazepines, a class of drugs that is prone to the development of tolerance upon chronic administration. Changes in $GABA_AR$ regulation might underlie this phenomenon. It is therefore important to understand the basic trafficking of $GABA_AR$ in neurons and how benzodiazepines might affect this.

	AMPA R		GABA R	
	GluR1/4	GluR2/3	β1	β3
phosphorylation	by PKA at Ser 842 and 845, by CamKII at Ser831 (Lee 2000, Ehlers 2000, Esteban 2003)	by PKC at Ser880 (McDonald 2001, Seidenmann 2003)	by PKA at Ser 409 (Hinkle 2003) by PKC at Ser 409 (Krishek 1994)	by PKA at Ser408/409 (Hinkle 2003) by PKC at Ser 409 (Brandon 2000)
dephosphorylation	by calcineurin at Ser 845 (Ehlers 2000, Snyder 2003)			PP2A (Jovanovic 2004)
interaction with proteins	SAP97 (Wu 2002) TARPs (Tomita 2004, Bredt 2003)	membrane clusters: NSF (Noel 1999, Braithwaite 2002, Fu 2003) GRIP/ABP (Seidenmann 2003) PICK-1 (Chung 2000,Seidenmann 2003) stargazin (Tomita 2004) internal pool: GRIP/ABP (Braithwaite 2002, Fu 2003, Daw 2003)	RACK-1 (Brandon 2002)	Gephyrin (indirect) (Kannenberg 1997, Meyer 2000) Membrane clusters: Plic-1 (Bedford 2001) Internal pool: Plic-1 (Bedford 2001) RACK-1 (Brandon 2002) Rac1(Meyer 2000) GABA-RAP (Kneussel)
endocytosis induction	AMPA (Beattie 2000, Lin 2000) NMDA (Beattie 2000,Carroll 1999, Ehlers 2000) Glutamate (Chao 2002, Lissin, Tomita 2004)	AMPA (Braithwaite2002, Lin 2000) NMDA(Ehlers 2000)		BDNF (Brünig 2002, Jovanovic 2004) muscimol (Meyer 2000)
endocytosis inhibition	Ca^{2+} chelators, PP2AB/PP1 (Ehlers 2000)			
insulin	endocytosis GluR1 (Beattie 2000, Lin 2000)	↑ endocytosis (Man 2000)	↑ exocytosis, β2-dependent (Wan 1997)	
D1 R agonist	phosphorylation PKA site, ↑ surface GluR1 (Chao 2002)		↓ GABA currents via PKA/DARPP-32/PP1 (Flores-Hernandez 2000)	

2 Aim of the thesis

The number of $GABA_AR$ in the synapse determines the effectiveness of fast inhibitory neurotransmission. Changes in the amount of synaptic receptors numbers might underlie several patho-physiological states, as shown by an increase of $GABA_AR$ in the postsynaptic density in a kindling model of epilepsy (Nusser et al., 1998). It has been hypothesized that chronic diazepam might reduce the number of cell-surface receptors, thereby inducing tolerance. It also has to be taken into account that there are several $GABA_AR$ subtypes, which could be differentially affected by chronic diazepam treatment.

Insight in the dynamic regulation of $GABA_AR$ will give us a better understanding how tolerance occurs. The aim of the thesis was thus to investigate normal regulation of $GABA_A$ R in cultured hippocampal cultures with fluorescence microscopy and in cortical neurons with biochemical methods. Secondly, using point-mutated mice available in the Institute, we analysed the role of different $GABA_A$ R subtypes in mice for the development of sedative tolerance after chronic diazepam or zolpidem treatment.

Publication 1: Subsynaptic localisation of internalised $GABA_A$ receptors (submitted to European Journal of Neuroscience)

Endocytosis of $GABA_AR$ can be induced by factors such as BDNF (Brünig 2002), or by drugs like benzodiazepines (Tehrani & Barnes 2001, Ali & Olson 2001). Most of this work was done in heterologous expression systems or with biochemical methods. However, no reports are available so far for concerning the subcellular localization of $GABA_A$ R after endocytosis. We tagged the surface receptors in living cells with antibodies and observed the distribution and localization of surface and internalized tagged $GABA_AR$ in cultured hippocampal neurons using gephyrin as a GABAergic postsynaptic marker. We inhibited clathrin-coated vesicle endocytosis using various procedures to determine how $GABA_AR$ are internalized. The results obtained with high resolution fluorescence microscopy were verified in cultures of cortical neurons with a biotinylation assay on western blots.

Publication 2: Requirement of α5-$GABA_A$ receptors for normal development of tolerance to the sedative action of diazepam (Journal of Neuroscience, 2004; 24(30): 6785-90).

A major class of drugs acting on $GABA_AR$, the benzodiazepines, has as a drawback that tolerance to their actions can develop readily after chronic use. It is unclear what the molecular mechanisms behind this tolerance are. The general consensus is that no substantial changes in benzodiazepine binding sites occur. A question that is still open is whether all $GABA_AR$ participate in the development of tolerance. Specific neuronal circuits, containing different $GABA_AR$ subtypes, mediate the spectrum of action of

diazepam, suggesting that specific $GABA_AR$ subtypes could mediate the development of tolerance. To investigate the contribution of various $GABA_AR$ subtypes for the development of sedative tolerance, we used mice in which diazepam binding to specific $GABA_AR$ subtypes was eliminated by a point mutation. We assessed the motor depressing actions of a test dose of diazepam after an 8-day diazepam treatment schedule in wild type, α1(H101R), α2(H101R), α3(H126R) and α5(H101R) mice. We also examined possible changes in the number of total benzodiazepine binding sites and of α5-specific binding sites in tolerant versus non-tolerant mice using autoradiography to determine whether chronic drug treatment alters the expression of functional $GABA_AR$.

Publication 3: Selective enhancement of α1-$GABA_A$ receptor activity by zolpidem is insufficient to induce full sedative tolerance and hippocampal α5-$GABA_AR$ downregulation (in preparation).

The development of sedative tolerance to diazepam appeared to depend on concurrent activation of α1- and α5-$GABA_AR$. This would imply that compounds, such as zolpidem, that act only on α1-$GABA_AR$ subtypes would have a lower tolerance liability. Zolpidem has a high affinity for α1-$GABA_AR$ subtypes, an intermediate affinity for α2- and α3-$GABA_AR$ subtypes and a low affinity for α5-$GABA_AR$ subtypes. Due to this affinity pattern, chronic zolpidem is also able mimic the effect of diazepam seen in chronically diazepam-treated α5(H101R) mice. These mice did not display sedative tolerance, suggesting that the α1-$GABA_AR$ are still functional, however, we could not measure α5 binding sites in these mice due to the point mutation.

We wanted to confirm that specific enhancement of α1-mediated inhibition is insufficient for the development of sedative tolerance and the associated changes in α5-$GABA_AR$ binding sites. We assessed the motor depressant effect of zolpidem after chronic zolpidem administration and measured the number of α5-$GABA_AR$ binding sites in the hippocampus of these mice. Furthermore, we also tested whether α1-$GABA_AR$ are still pharmacologically, active by injecting a test dose of zolpidem after diazepam treatment. A time course of motor activity was also measured directly after the injection of the last dose to assess the duration of the sedative effect of zolpidem in chronically treated mice

3 Results

3.1 Internalized GABA-receptor subunits are transferred to an intracellular pool associated with the postsynaptic density.

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Abstract

Endocytosis represents an important mechanism regulating cell-surface expression of neurotransmitter receptors, including $GABA_A$ receptors, in neurons. Little is known, however, about trafficking of internalized receptors. Here, we used antibody tagging in living hippocampal neurons in culture to monitor $GABA_A$ receptor internalization. We show that cell-surface receptors have a homogeneous distribution reflecting their mobility in the membrane. Unexpectedly, internalized $GABA_A$ receptors were detected in a subsynaptic pool associated with gephyrin at postsynaptic sites, whereas AMPA-receptors were accumulated in the soma. This process was time-dependent and could be prevented by blocking clathrin-coated vesicle endocytosis. In control experiments, the existence of an intracellular pool of $GABA_A$ receptors associated with gephyrin was confirmed independently of internalization of surface receptors, and constitutive endocytosis could be demonstrated for both AMPA- and $GABA_A$ receptors using a biotinylation assay. These results suggest that cycling of $GABA_A$ receptors between the cell surface and the subsynaptic pool provides a mechanism for short-term regulation of GABAergic neurotransmission.

Introduction

$GABA_A$ receptors ($GABA_AR$) are ligand-gated ion-channels that mediate the major part of inhibitory neurotransmission in the brain and are a target for several classes of drugs, such as barbiturates and benzodiazepines (Fritschy and Brunig, 2003). A functional receptor generally consists of a pentameric complex containing 2α, 2β and 1γ subunit selected from a repertoire of multiple variants (α1-6, β1-4, γ1-4) (Barnard et al., 1998). Most $GABA_AR$ that are clustered at postsynaptic sites contain the γ2 subunit and are colocalized with gephyrin, a cytoplasmic protein interacting with the cytoskeleton (Sassoe-Pognetto et al., 2000; Luscher and Keller, 2004). The amount of $GABA_AR$ present in the postsynaptic density is cell type-dependent and correlates directly with synaptic strength, as shown under normal conditions (Nusser et al., 1997) and in a model of temporal lobe epilepsy (Nusser et al., 1998). Cell-surface expression and synaptic trafficking of $GABA_AR$ are regulated by phosphorylation mechanisms (Connolly et al., 1999a) and by factors such as BDNF (Brunig et al., 2001; Jovanovic et al., 2004) and insulin (Wan et al., 1997), which activate tyrosine kinase receptor-dependent signaling pathways.

In a recombinant expression system, cell-surface expression of $GABA_AR$ was reduced upon chronic diazepam exposure, an effect due to increased endocytosis and dependent on PKA activation (Ali and Olsen, 2001). *In vivo,* $GABA_AR$ endocytosis likewise is enhanced following chronic ethanol (Kumar et al., 2003) or chronic flurazepam treatment (Tehrani and Barnes, 1997). Constitutive endocytosis mediated by clathrin-coated vesicles has been proposed as a mechanism regulating cell-surface expression of $GABA_AR$. It was first demonstrated in HEK cells transfected with α1β2γ2 subunit cDNAs (Connolly et al., 1999b). In this assay, $GABA_AR$ were detected on the surface as well as in a perinuclear compartment and were shown to shuttle between the two pools. Additional evidence was provided by Herring et al. (2003), who demonstrated that the clathrin adaptor-protein AP-2 can associate with the β-subunit and disruption of this interaction results in an increased response to application of GABA. Likewise, overexpression of a dominant-negative form of dynamin prevents $GABA_AR$ internalization (Herring et al., 2003). Similar mechanisms are also operative in neurons (Kittler et al., 2000), as demonstrated by blocking the ampiphysin-dynamin interaction with an inhibiting peptide.

Data from transfected Xenopus oocytes also suggested that $GABA_AR$ present in an intracellular compartment could be delivered back to the surface (Filippova et al., 2000). On the other hand, modulation by PKC seems to prevent internalized receptors from returning to the surface (Connolly et al., 1999a). Association of internalized $GABA_AR$ with the ubiquitin-like protein Plic-1 was suggested to prevent their degradation by the proteasome (Bedford et al., 2001). Plic-1 might therefore stabilize the intracellular pool of receptors and facilitate their reinsertion into the membrane.

Although several mechanisms involved in $GABA_AR$ endocytosis have been characterized, little is known about the localization and fate of internalized $GABA_AR$, in particular in dendrites. The aim of the present study was to monitor internalized $GABA_AR$ with immunocytochemical methods in primary hippocampal cell cultures, using antibodies to tag cell surface receptors. The results show that most internalized $GABA_AR$ are relocated to a subsynaptic pool associated with gephyrin at postsynaptic sites, suggesting a novel mechanism for short-term regulation of GABAergic synaptic function by rapid exchange of cell-surface receptors.

Material & Methods

Animals. Rat embryos were obtained from time-pregnant Wistar rats (Harlan, Horst, the Netherlands). All experiments were approved by the cantonal veterinary office of Zurich.
Reagents. All reagents were from Fluka (Buchs, Switzerland) unless stated otherwise. The dynamin-inhibiting peptide (Shupliakov et al., 1997) QVOSRNPNRAP (non-permeable) and Myr-QVOSRNPNRAP (membrane permeable) were from Eurogentech (Seraing, Belgium). Sulfo-NHS-SS-biotin and Ultralink Immobilized Neutravidin biotin-binding protein were from Pierce (Rockford, IL). Buffer A was a 25mM Hepes buffer supplemented with 30 mM glucose, 119 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 μM glycine and 500 nM TTX (modified from Archibald et al., 1998). The solubilization buffer contained 0.5% deoxycholate, 1% NP40, 0.1% SDS, 150 mM NaCl, 50 mM Tris, 2 mM EGTA, 1 mM EDTA, 0.02% NaN_3, 10 μg/ml leupeptin, 1 ml/l aprotinin, 0.1 mM PMSF.
Cell culture. Primary cultures of E18 hippocampal neurons and E19 cortical neurons were prepared as described previously (Brunig et al., 2002b). Hippocampus or cerebral cortex was dissected on ice and incubated for 15 min at 37°C in PBS, pH 7.4, containing 1 mg/ml BSA, 10 mM glucose, 0.5 mg/ml papain (Sigma, St. Louis, MO) and 10 μg/ml DNAse-1 (Sigma). Neurons were then dissociated by gentle trituration with a fire-polished Pasteur Pipette and suspended in DMEM (Gibcom, Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (Invitrogen, San Diego, CA). The hippocampal neurons were plated at a density of 15'000 cells/cm^2 and the cortical neurons at a density of $2.5*10^6$cells per Petri dish (60 mm). The medium was exchanged after 1 day with a defined, serum-free medium (Brewer and Cotman, 1989), and coverslips with glial cells, prepared from P0 rat cortex, were placed on top of the hippocampal cultures. No glia were added to the cortex cultures. Cultures were kept in a humidified incubator at 37° and 10% CO_2 and were used after 2-3 weeks.
Antibodies. Distinct subtypes of $GABA_AR$ were visualized by immunofluorescence staining using subunit-specific antibodies raised in our laboratory against N-terminal epitopes (α1, α2, γ2). These antibodies have been characterized extensively (Fritschy et al., 1992; Fritschy and Mohler, 1995; Nusser et al., 1996). In addition, the monoclonal antibody bd-17, recognizing both the β2 and β3 subunits, was used (Ewert et al., 1990). The

monoclonal antibody mAb7a against gephryin was supplied by Connex (Martinsried, Germany). Rabbit anti-GluR1 was from Chemicon (Temecula, CA). Mouse anti-GluR1 against an extracellular epitope of GluR1 (mAb8E11, GSALRNPVN (GluR1-flop740-748) plus a partial epitope, QGLL (GluR1-flop757-760), (Tonnes et al., 1999)) was a kind gift of Prof. Peter Streit (Brain Research Institute, University of Zurich, Switzerland).

Distinction of cell-surface and intracellular $GABA_A$ receptors. Cell-surface receptors were labeled by a brief incubation of living hippocampal cultures for 10 min at room temperature (RT) with a high concentration of rabbit antibody against the α1 subunit (1:400 in buffer A containing 0.5 M sucrose) and quickly rinsed. These conditions are non-permissive for internalization. The cultures were then fixed and permeabilized with methanol for 10 min at –20°C and rinsed with PBS. With this treatment, intracellular receptors became accessible to primary antibodies. The cultures were then incubated for 60 min at RT in a guinea pig anti-α1 subunit antiserum (diluted 1:4000 in PBS containing 10% FCS), washed three times with PBS, and incubated for 30 min in a mixture of goat anti-rabbit and goat anti-guinea pig secondary antibodies conjugated to Alexa 488 (Molecular Probes, Eugene, OR) and Cy3 (Jackson Immunoresearch, West Grove, PA), respectively.

Internalization protocol. Cell-surface receptors were tagged in living cultured neurons with primary antibodies against the α1, α2, or γ2 subunit. The tagged receptors were allowed to internalize at 37ºC, prior to fixation and permeabilization of the cultures and subsequent labeling of internalized receptors and intracellular markers, such as gephyrin. The experiments started by removing the medium and equilibrating the cultures in buffer A/0.5 M sucrose to inhibit internalization. They were then incubated with guinea pig antisera against the α1 (1:400), α2 (1:100) or γ2 subunit (1:200) for 10 min at RT in buffer A/0.5 M sucrose to tag the cell-surface receptors. After washing with Buffer A/0.5M sucrose, the medium was returned to the cultures, which were placed back into the incubator at 37ºC for up to 60 min to allow internalization of the tagged $GABA_AR$. The cells were then fixed for 10 min under non-permeabilizing conditions (4% paraformaldehyde in 0.1 M phosphate buffer containing 4% sucrose). They were rinsed with PBS and incubated for 30 min with goat anti-guinea pig antibodies conjugated to Alexa488 to visualize the remaining surface receptors. After washing with PBS, cells were permeabilized with methanol at –20 °C for 10 min and incubated with mAb7a against gephyrin (1:400) for 60 min. and washed again. Finally, the cultures were incubated for 30 min with a mixture of two secondary antibodies (goat anti-guinea pig conjugated to Cy3 to label the internalized receptors and goat anti-mouse conjugated to Cy5 for gephyrin). After washing, cultures were dried and coverslipped. In control experiments, the internalization of tagged receptors was prevented by incubating the cultures at 4ºC or with 0.5 M glucose. The involvement of clathrin-coated vesicles was assessed using a membrane-permeable, myristoylated dynamin-inhibiting peptide (Shupliakov et al., 1997) and the non-myristoylated control peptide, which were

added to the medium 30 min before antibody labeling (50 μM in PBS). The same medium with peptide was applied for the internalization.

For comparison with $GABA_AR$, the internalization of AMPA receptors containing the GluR1 subunit was visualized with a similar protocol, using a monoclonal antibody raised against an extracellular epitope. The medium was supplemented with 100 μm AMPA to enhance the rate of internalization (Beattie et al., 2000).

Biotinylation assay. Biotinylation assays were performed as described previously (Mammen et al., 1997) to assess internalization of $GABA_AR$ independently of antibody tagging. Leupeptin (10 μg/ml), a lysosomal inhibitor, and/or the myristoylated dynamin inhibiting peptide (50 μM) were added to the medium 30 min before the start of the experiment. The cerebral cortex cultures were placed on ice and biotinylated with ice-cold Sulfo-NHS-SS-biotin (0.5 mg/ml in Buffer A) for 12 min and washed with ice-cold buffer A. Cultures for the total surface and background measurement were left on ice, and other cultures were returned to the incubator for 30 min. In all samples except the 'total surface', biotin was cleaved with a gluthathione solution (75 mM gluthathione, 75 mM NaCl, 10 mM EDTA, 1% BSA, pH 8.0) for 2 x 15 min on ice. After solubilization, biotinylated proteins were precipitated using Neutravidin beads, eluted with sample buffer and resolved by SDS-PAGE. Samples were immunoblotted with a rabbit antibody against the GluR1 subunit (1:4000) and $GABA_AR$ β2/3 subunits (monoclonal bd-17; 1:4000), followed by secondary antibodies conjugated to horseradish-peroxidase (1:5000) and subsequent signal detection with luminol/enhancer buffer (Bio-Rad Laboratories, Reinach Switzerland).

Data analysis. Images from immunofluorescence staining were digitized with a high-resolution digital camera (Hamamatsu Orca, Hamamatsu Photonics, Hamamatsu City, Japan) driven by the OpenLab imaging software (Improvision, Coventry, England). For quantitative analysis of staining intensity, images were acquired with a laser scanning confocal microscope (LSM 510 Meta, Carl Zeiss AG, Feldbach, Switzerland), using a 100x objective with a numerical aperture of 1.4 (voxel size, 0.18 μm). Image acquisition parameters were adjusted to the full dynamic range of the photodetector and all images from a particular experiment were taken with the same settings. Cells to be imaged were chosen on their overall appearance and contained average densities of gephyrin clusters. Per cell culture, at least 10 fields were sampled. Fluorescence signals were calibrated using fluorescent beads (Molecular Probes, Eugene, OR) of known relative intensity. Gephyrin clusters were used as a mask to outline α2-$GABA_AR$ clusters, and the size and fluorescence intensity were integrated for all clusters in the field, using a threshold segmentation algorithm (MCID M5 software; Imaging Research, Ste-Catherines, ON). To be considered, clusters had to have a size of at least 3 pixels and 15% intensity above background (equaling an intensity of 5% on the calibration curve ranging from 0-35 %). The data for

the dynamin inhibiting peptide quantification were collected from three independent experiments.

Statistics. Results, expressed as mean ± SD, were analyzed using non-parametric Kruskall-Wallis analysis and, whenever appropriate, Dunn's Multiple comparison's test for post-hoc mean comparisons.

Results

Identification of two distinct pools of $GABA_A$ receptors.

To distinguish cell-surface receptors from intracellular receptors in untreated, mature hippocampal neurons in vitro, cells were labeled sequentially with a rabbit and a guinea pig antiserum against the α1 subunit before and after membrane permeabilization. Cell-surface receptors were labeled under conditions non-permissive for internalization to prevent their translocation into the cell prior to labeling the existing intracellular pool. Staining of gephyrin was used to mark the GABAergic postsynaptic densities (Brunig et al., 2002a). The analysis was performed in presumptive interneurons, which express an intense staining for the α1 subunit in cultures after 14-21 days-in-vitro DIV. As reported previously (Brunig et al., 2002a), most pyramidal cells in the same culture exhibited only a very weak staining for the α1 subunit (not shown).

The staining of cell-surface α1-$GABA_AR$ in these interneurons was homogeneous on the soma and neurites (Fig. 1A, green), with little evidence for local aggregation in the form of clusters. Although co-localization with gephyrin-immunoreactivity (-IR) was evident (Fig. 1, arrows), the distribution pattern of the α1 subunit could not be used to predict the localization of GABAergic synapses in these cells. Staining of the intracellular α1-$GABA_AR$ with the guinea pig antibody (Fig. 1B, red) was detected in exactly the same cells. Most unexpectedly, the corresponding α1 subunit-IR exhibited a clustered pattern in neurites, and these clusters were colocalized extensively with gephyrin. Only a few intracellular receptor clusters did not appear to be localized at synaptic sites (Fig. 1, arrowhead). This observation implies that, although many α1- $GABA_AR$ are present in the membrane of interneurons, there is, in addition, an intracellular pool of $GABA_AR$ associated with gephyrin presumably at postsynaptic sites. Furthermore, this intracellular pool is present only in cells exhibiting a prominent surface staining.

In control experiments, the rabbit antibodies bound to the surface receptors were saturated with unlabeled secondary antibodies prior to permeabilization and application of fluorescent anti-rabbit IgGs. No staining was observed (not shown), confirming that internalization was blocked effectively. Conversely, gephyrin staining was not detectable prior to fixation and permeabilization of the cells (not shown), indicating that membrane integrity was preserved during the tagging procedure.

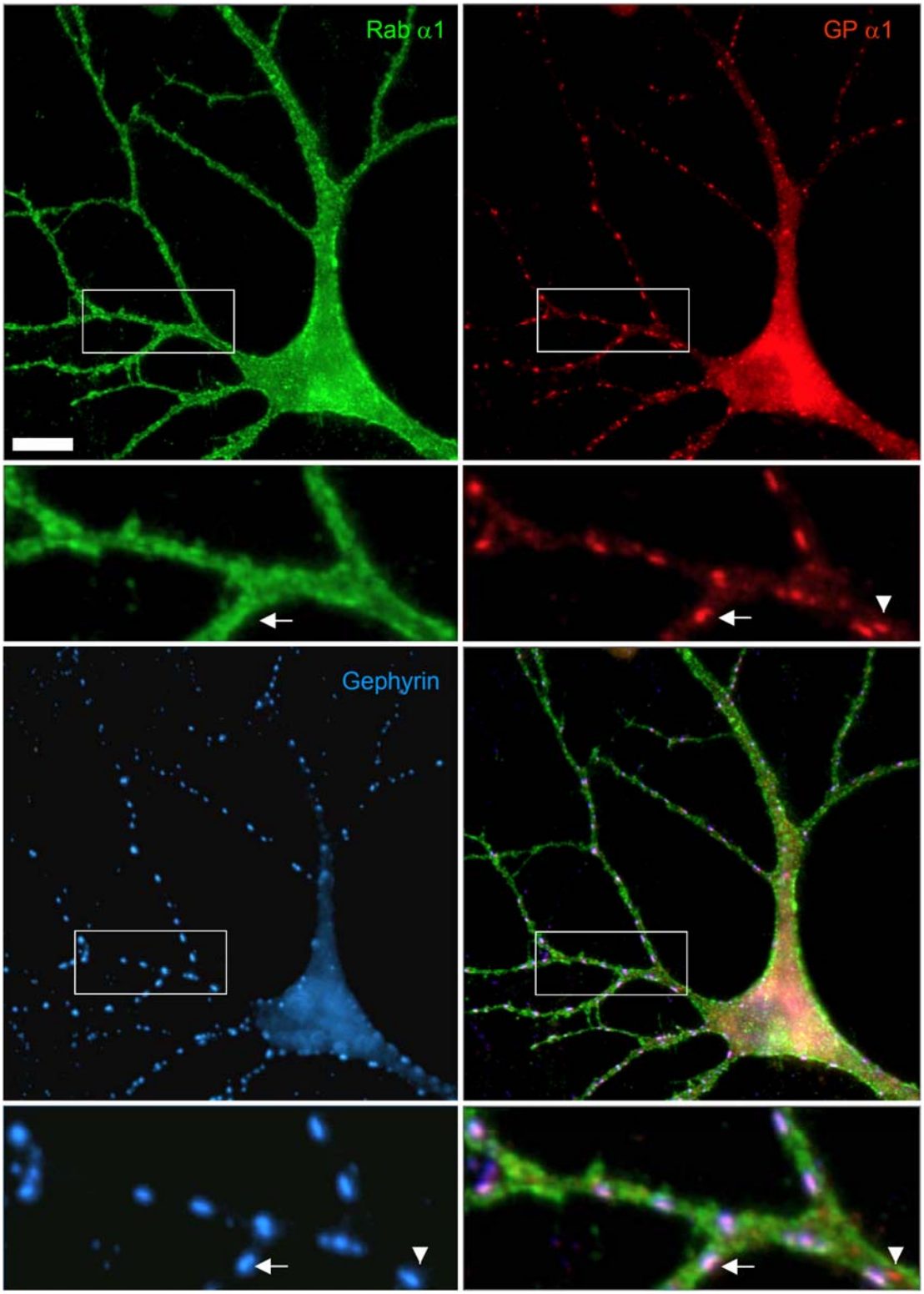

Fig. 1. Digital photomicrographs from epifluorescence microscopy illustrating the distinct distribution of cell-surface (green) and internal (red) $GABA_AR$ in a cultured hippocampal neuron, as revealed by sequential labeling with α1 subunit antibodies raised in distinct species before and after membrane permeabilization. The surface receptors, detected with the rabbit antibody, exhibit a homogeneous distribution on dendrites, whereas the internal receptors (guinea pig antibody) are clustered at sites containing gephyrin (blue). The boxed area is enlarged in the bottom row. Arrows show the presence of cell surface α1 subunit staining at a presumptive postsynaptic site labeled with gephyrin. A cluster of internal receptors is present at the same site, precisely colocalized with gephyrin. Arrowheads point to a cluster of internal α1 subunit staining not associated with gephyrin. Scale bar, 10 μm.

Internalized $GABA_A$ receptors replenish the intracellular pool

We investigated the possibility that the intracellular pool of $GABA_AR$ associated with gephyrin originates from surface receptors by monitoring the distribution of internalized receptors that were previously tagged at the cell surface. Live cultures were incubated with primary antibodies, washed, and returned to the incubator for one hour to allow for constitutive (or antibody-induced) endocytosis. Cell-surface receptors were visualized with fluorescent secondary antibodies after fixation with 4% PFA/sucrose, whereas internalized receptors were detected subsequently, using a different fluorochrome, after permeabilization of the membrane. These experiments were performed with the α1 subunit in interneurons and with the α2 and γ2 subunit in principal cells after 14-21 DIV (Fig. 2). In interneurons, staining of surface α1-$GABA_AR$ was again homogeneously distributed and showed no enrichment at postsynaptic sites. In contrast, the receptors tagged at the surface and allowed to internalize formed clusters in neurites that were colocalized extensively with gephyrin with a characteristic synaptic pattern (Fig. 2A). Similar results were observed also for the α2- and γ2- subunit in presumptive pyramidal cells (Fig. 2B and C), with a diffuse distribution of cell surface receptors, and a clustered distribution of internalized receptors. The fact that brightly stained clusters co-localized with gephyrin become visible only after permeabilization of the membrane is harmonious with their intracellular localization.

The appearance of the intracellular clusters was time-dependent and correlated with the duration of incubation at 37°C. At time zero, the Cy3-conjugated secondary antibody used to label internalized receptors apparently cross-reacted with the Alexa 488-conjugated antibody applied onto cell-surface receptors, resulting in a similar staining pattern (Fig. 3A). This cross-reactivity diminished gradually, as internalized receptors became visible after 20 min at 37°C (Fig. 3B). The staining intensity of the intracellular clusters increased further with time (Fig. 3C-E) and after 30 min incubation, the cross-reactivity between the two secondary antibodies was negligible. These experiments were repeated in the presence of a GABA agonist (THIP, 150 μM), an antagonist (bicuculline, 40 μM), or an allosteric modulator (diazepam, 1 μM), but these treatments during the incubation did not affect the speed or extent of internalization, as assessed by the appearance and staining intensity of intracellular clusters (data not shown).

These data indicate that α1-, α2- and γ2-$GABA_AR$ are internalized in a time-dependent manner and become localized in the subsynaptic pool associated with gephyrin, suggesting that this intracellular pool is replenished by $GABA_AR$ internalization. To verify that these results are not an artifact of the staining procedure, similar experiments were performed for AMPA receptors, using an antibody against an extracellular epitope to tag cell surface receptors in living cells, and sequential detection of internalized receptors after 15 min incubation at 37°C in the presence of 100 μM AMPA.

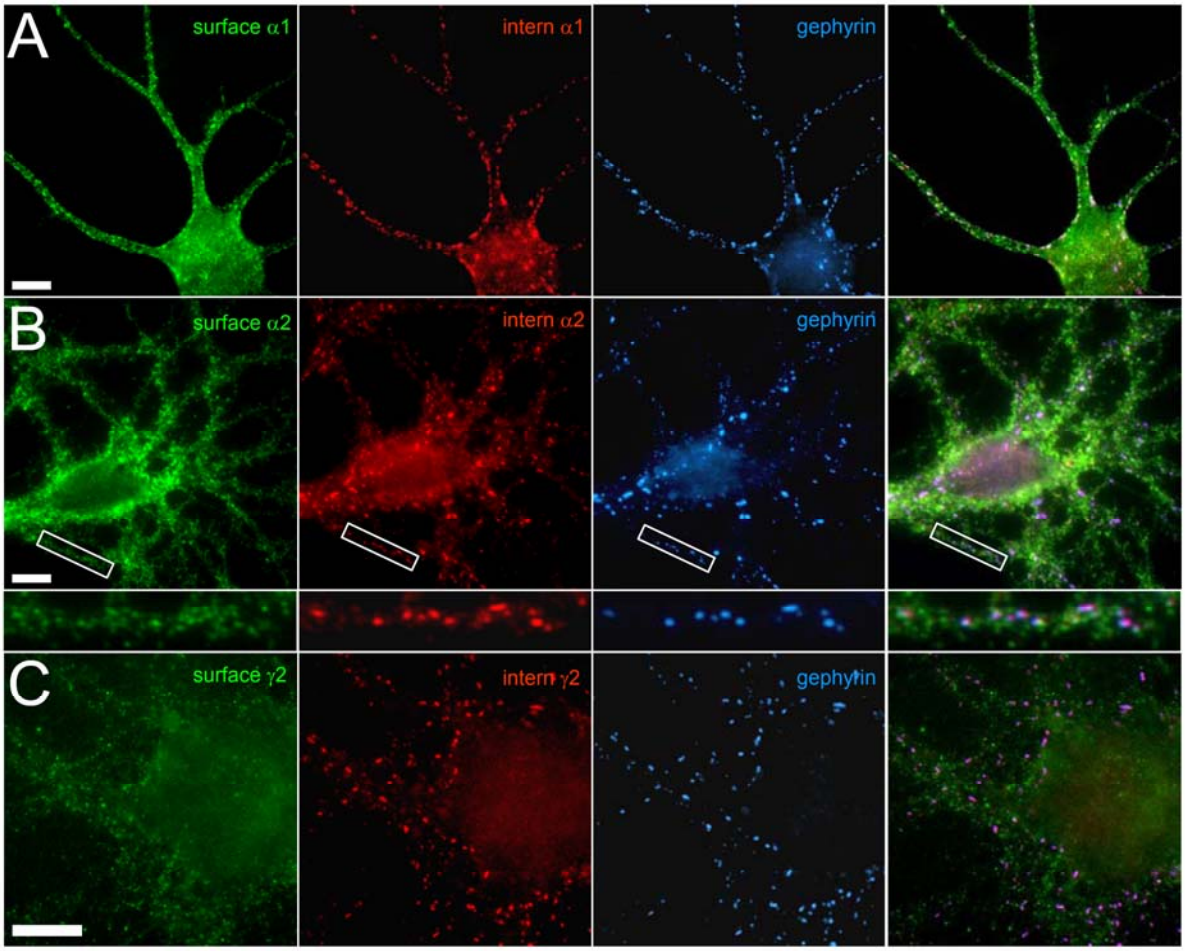

Fig. 2. Digital photomicrographs from epifluorescence microscopy demonstrating the distribution of $GABA_AR$ clusters (red) following internalization of surface receptors tagged with an antibody in living cultures maintained at 37ºC for 60 min. Upon fixation, cell-surface receptors (green) appear uniformly distributed, whereas the internalized receptors (red), detected after membrane permeabilization, are distributed in clusters colocalized with gephyrin (blue). Merged images are shown in the panels on the right. A) Internalization of α1 subunit-containing $GABA_AR$ in a presumptive interneuron; B) Internalization of α2 subunit-containing $GABA_AR$ in a pyramidal cell; the boxed area is enlarged below to illustrate the spatial relationship of the surface receptors and internalized receptors with gephyrin. C) Internalization of γ2 subunit-containing $GABA_AR$ in a pyramidal cell. Scale bars: A-C, 10 μm.

In these experiments, images were collected by confocal laser scanning microscopy to distinguish the surface and the interior of the cells. These experiments were directly compared to results obtained for the $GABA_AR$ α2 subunit. In Fig. 4, two confocal planes across the same cell are shown for each antibody, depicting cell surface receptors in green and internalized receptors in red. At the two levels, internalized AMPA receptors accumulate within the cell body and dendrites within 15 min (Fig. 4A), without forming clusters at presumptive synaptic sites. In contrast, the internalized α2-$GABA_AR$ subunit was redistributed to clusters in dendrites, and was only moderately accumulated in the soma, as seen after 60 min of internalization (Fig. 4B). Therefore, the selective aggregation of internalized $GABA_AR$ in a pool associated with gephyrin appears to be a specific property of these receptors.

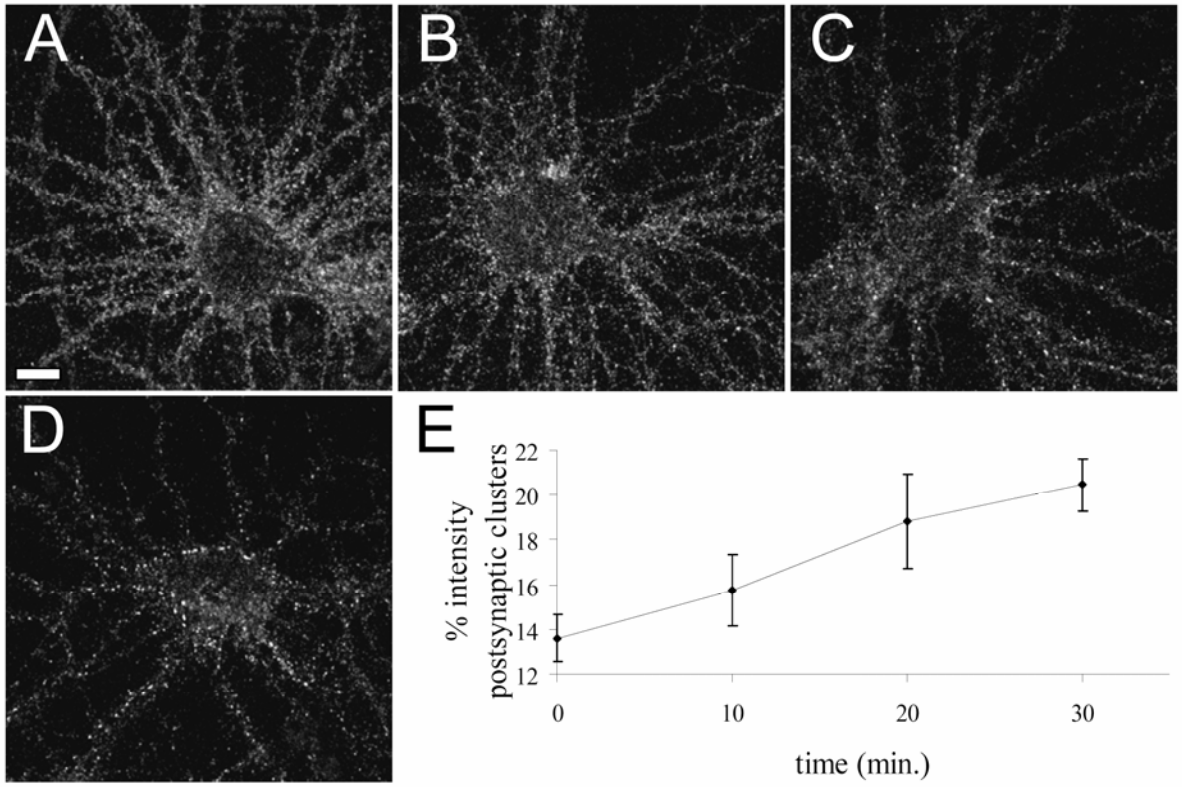

Fig. 3. Time-dependency of the internalization of cell surface $GABA_AR$ tagged with an antibody against the α2 subunit in living cells incubated at 37ºC for 0 (A), 10 (B), 20 (C), and 30 min (D). Antibodies bound to cell surface receptors were saturated with an unlabeled secondary antibody prior to applying the fluorescent antibody to detect internalized receptors. Images were collected by confocal laser scanning microscopy and a single confocal layer, close to the bottom of the cells, is depicted in each panel. At t0 and t10, a diffuse staining is apparent with the fluorescent secondary antibody, but clusters appear gradually thereafter and become clearly discernable after 20 min (C) and are quite prominent after 30 min incubation (D). Quantification of the intensity of the intracellular α2 subunit fluorescence at post-synaptic sites at the different time-points is depicted in **E.** Scale bar, 10 μm**.**

Blocking clathrin-coated vesicle-mediated endocytosis inhibits the internalization of $GABA_A$ receptors.

The internalization of $GABA_AR$ noted above was time-dependent, but it was not affected by a GABA agonist or antagonist, raising the possibility that it was triggered by antibody binding. Therefore, several experiments were performed to test whether constitutive internalization mediated by a clathrin-dependent mechanism occurs in pyramidal cells, using the α2 subunit staining. Hypertonic sucrose inhibits the formation of clathrin-coated vesicles by flattening the clathrin lattices (Heuser and Anderson, 1989) and can therefore be used to inhibit internalization. Alternatively, clathrin-coated vesicle-mediated endocytosis can be blocked by competitive blockade of the interaction between dynamin and ampiphysin, using a dynamin-interacting peptide (Shupliakov et al., 1997). Cultures that were incubated at 4°C for one hour did not show any clear clusters of internalized receptors (Fig. 5B) compared to control (Fig. 5A), providing the first evidence for an active biochemical process. The addition of hypertonic sucrose also inhibited the formation of intracellular clusters (Fig. 5C), to the same extent as seen in cultures that were kept at 4°C.

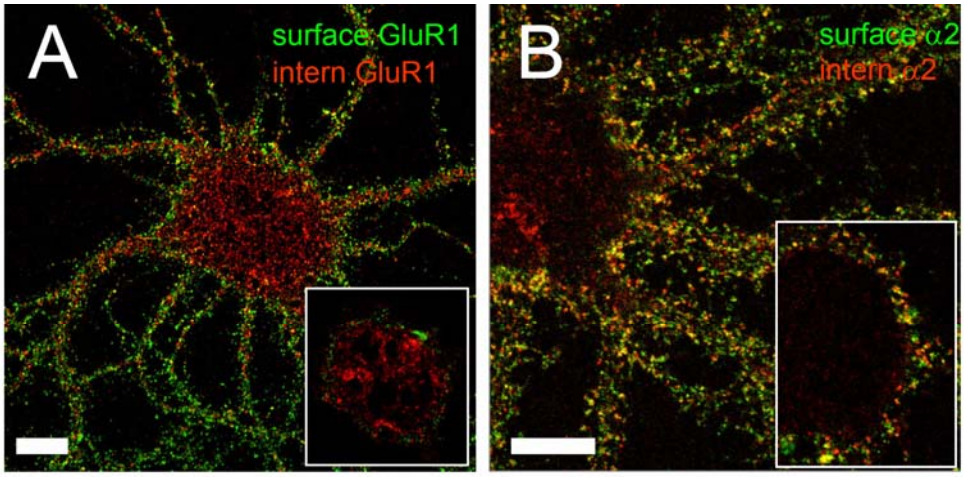

Fig. 4. Differential subcellular distribution of internalized AMPA receptors (GluR1 subunit) and $GABA_AR$ (α2 subunit) in cultured hippocampal cells, as illustrated by confocal laser scanning microscopy following labeling of cell-surface receptors (green) and internalized receptors (red) upon incubation at 37ºC for 15 and 60 min, respectively. A single confocal image close to the bottom of the cell is depicted; the insets show a confocal plane higher up through the soma of the same cell. Staining for internalized GluR1 subunit is most prominent in the soma, whereas internalized α2 subunit-containing $GABA_AR$ mainly are present in clusters on dendrites and are much less prominent in the cell body. Scale bars, 10 μm.

Finally, when a membrane-permeable, myristoylated dynamin-inhibiting peptide was added to the medium 30 min prior to antibody tagging and during the internalization step, the staining intensity of internalized α2 subunit clusters was greatly reduced (Fig. 5E). The non-membrane permeable form of the peptide (Fig. 5D) did not prevent the formation of internalized clusters. The staining intensity of the postsynaptic, internalized α2 clusters was quantified using fluorescent beads with standardized intensities for calibration. The myristoylated dynamin-inhibiting peptide effectively reduced the intensity of the postsynaptic clusters (71% of vehicle treated cultures) [H=25.80, P<0.001, Kruskal-Wallis]. The non-permeable form did not affect the staining intensity. Intriguingly, cultures treated with the myristoylated peptide also showed a marked reduction in intensity of gephyrin clusters (81% of vehicle treated cultures) [H=25.36, ** P<0.01 Kruskal-Wallis]. Thus, blocking clathrin-coated vesicle endocytosis can inhibit the formation of the intracellular clusters in this immunocytochemical assay.

To determine whether $GABA_AR$ are constitutively internalized in mature, primary neuronal cultures, independently of antibody tagging, surface proteins were bound with a cleavable form of biotin. After incubation and subsequent endocytosis at 37°C, the biotin on cell-surface proteins was cleaved with glutathione and the remaining biotinylated proteins were precipitated with Neutravidin beads, separated by gel electrophoresis, and $GABA_AR$ subunits identified by Western blotting. The constitutive internalization of the AMPA receptor GluR1 subunit was taken as a positive control. Under basal conditions, a prominent signal was observed for the endocytosed GluR1 subunit, whereas only a band of moderate intensity appeared for the $GABA_AR$ β2/3 subunits (Fig. 5G). Only the β3 subunit, which

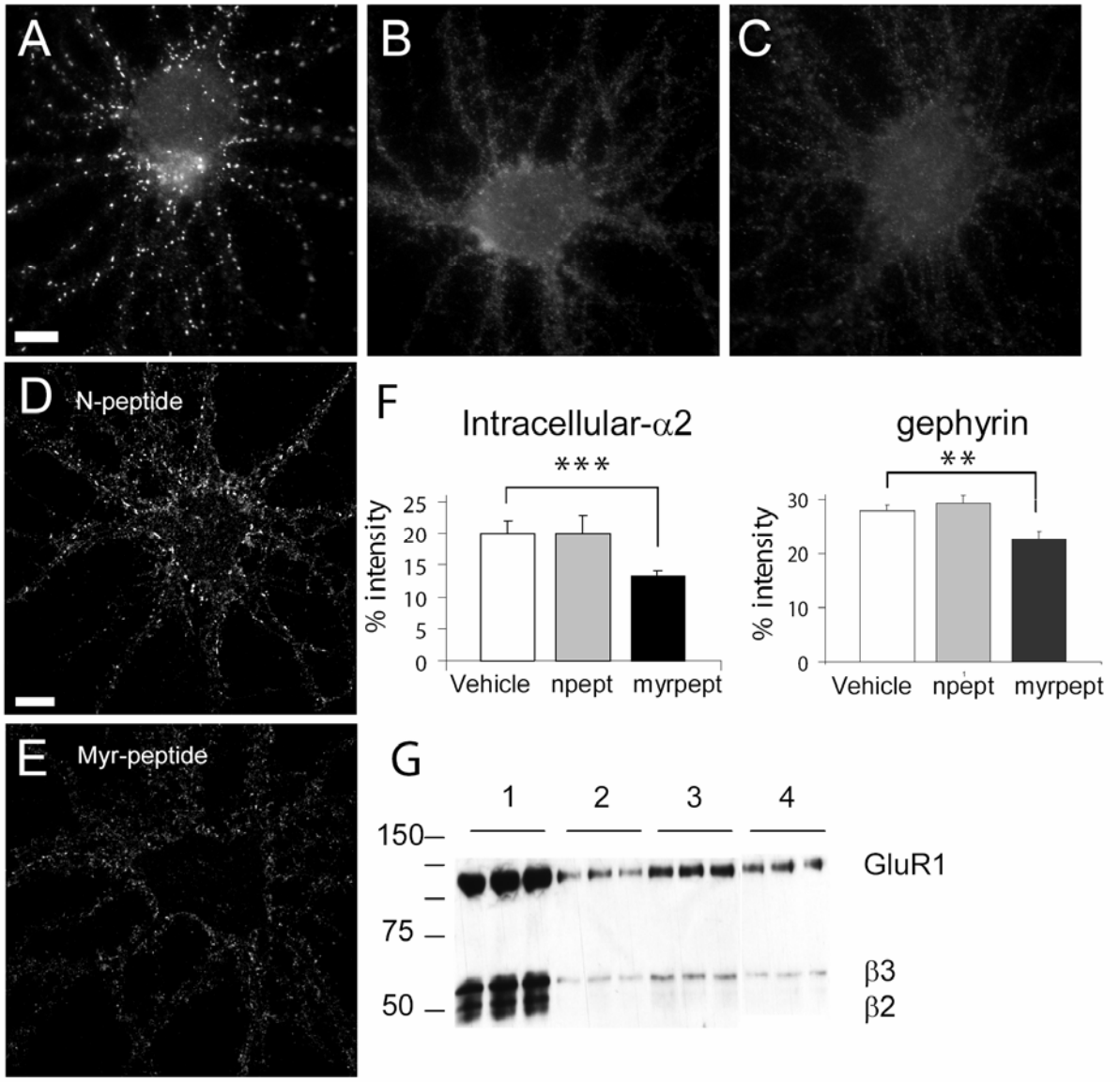

Fig.5. Inhibition of clathrin-coated vesicle formation prevents internalization of $GABA_AR$ and their appearance in intracellular clusters. **A-C**: Digital images from epifluorescence microscopy depicting cell-surface intracellular α2-$GABA_AR$ in hippocampal pyramidal cells that were incubated for 60 min at 37° (**A**; control), at 4°C (**B**), or at 37° in medium with 0.5 M sucrose (**C**). In the two latter conditions, no internalized receptors became apparent. Scale bar, 10 μm. **D, E**: Effect of 50 μM of a non-permeable (**D**) or membrane-permeable, myristoylated form of a dynamin-inhibiting peptide (**E**) on the internalization of α2 subunit-containing $GABA_AR$ tagged at the cell surface, as visualized by confocal laser scanning microscopy. Inhibition of the dynamin-ampiphysin interaction by the membrane-permeable peptide largely blocked the internalization of the tagged receptors. Scale bar, 10 μm. **F:** Quantification of the effect of the dynamin-inhibiting peptide on the staining intensity of internalized α2 subunit clusters and gephyrin clusters. The myristoylated peptide reduced the intensity of both α2 subunit (*** $P<0.001$; Kruskal-Wallis) and gephyrin (** $P<0.01$; Kruskal-Wallis) clusters, whereas the non-permeable form had no effect. This experiment was replicated three times with 15-30 cells measured per treatment. **G:** The membrane-permeable form of the dynamin inhibiting peptide blocks endocytosis of AMPA receptors and $GABA_AR$ in a biotinylation assay. Surface proteins and internalized proteins were separated by SDS-PAGE and labeled by Western blotting using antibodies to the GluR1 subunit (upper band) and the monoclonal bd-17 (lower bands). Lane 1: total surface; lane 2: background measured in cultures maintained at 4°C to prevent internalization; 3: internalized receptors following 30 min incubation at 37 °C, 4: internalized receptors following incubation for 30 min at 37 °C in the presence of 50 mM myristoylated dynamin-inhibiting peptide. Note that the upper of the two bands detected by bd-17, which corresponds to the β3 subunit is more strongly internalized than the lower band (β2 subunit).

was more abundant than the β2 subunit (upper band of the 50 kDa doublet in lane 1), was detectable inthe endocytosed sample (lane 3) following a short exposure of the film. The appearance of
these bands could be blocked effectively in the presence of the membrane-permeable dynamin-inhibiting peptide (Fig. 5G, lane 4), providing direct evidence for constitutive endocytosis of both AMPA receptors and $GABA_AR$ in primary neuronal cultures of the cerebral cortex.

Discussion

The present results demonstrate the existence in mature neurons of an intracellular pool of $GABA_AR$, which colocalize with gephyrin at presumptive postsynaptic sites. Cell-surface receptors tagged with antibodies in living cells have a rather homogeneous distribution on the soma and neurites and are constitutively internalized in clathrin-coated vesicles. After endocytosis, a large fraction of these receptors is transferred in the intracellular, subsynaptic pool of $GABA_AR$. The constitutive cycling of $GABA_AR$ between the cell surface and this subsynaptic pool likely represents a novel mechanism for short-term regulation of GABAergic synaptic plasticity.

Functional and ultrastructural studies provide unambiguous evidence that $GABA_AR$ are clustered postsynaptically opposite to GABAergic terminals (Somogyi 1998; Maccaferri et al., 2000; Bergersen et al., 2003). These receptors are associated with gephyrin, which is located selectively in the postsynaptic density of symmetric, inhibitory synapses (Giustetto et al., 1998; Sassoe-Pognetto et al., 2000). Extrasynaptic receptors are present at much lower density in the somatodendritic plasma membrane (Nusser et al., 1995). Owing to the narrowness of the synaptic cleft, postsynaptic $GABA_AR$ subunits are not readily accessible in fixed tissue to antibodies raised against extracellular epitopes. In electron microscopy, the problem has been circumvented by the use of post-embedding staining, where the synaptic cleft is exposed in ultrathin sections. In light microscopy, postsynaptic $GABA_AR$ clusters can be detected in sections prepared from weakly fixed, fresh frozen tissue, in which the integrity of cell membranes is compromised (Fritschy et al., 1998; Sassoe-Pognetto et al., 2000). The present results show that, in cultured hippocampal neurons, these postsynaptic clusters are not detected by immunofluorescence, unless the plasma membrane is permeabilized, indicating that a significant fraction of $GABA_AR$ at postsynaptic sites are not accessible to antibodies in intact cells. Therefore, $GABA_AR$ clusters seen in light microscopy, colocalized with gephyrin, most likely include also receptors located intracellulary. Labeling of cell-surface receptors revealed only minor differences in staining intensity between postsynaptic and extrasynaptic receptors. The reason for this discrepancy with ultrastructural studies is not known. Lateral movement of receptors diffusing from postsynaptic to extrasynaptic sites, as demonstrated for glycine and AMPA receptors

(Choquet and Triller, 2003), during the incubation with primary antibodies might account for this inconsistency, a process possibly amplified by “antibody capping”. Staining of cell-surface AMPA receptors (Fig. 4) also failed to demonstrate the presence of clusters in dendrites, possibly for the same reason.

A direct demonstration of the co-existence of the intracellular pool and surface receptors with a distinct distribution could be achieved in interneurons, using two antibodies raised in different species against the α1 subunit. It is very likely that the same compartmentalization also exists for α2-$GABA_AR$ in pyramidal cells, in view of the similarity of the distribution of internalized receptors in both cell types. Furthermore, the experiments with the γ2 subunit, which is present in most $GABA_AR$ subtypes, strongly suggest that heteromeric complexes are translocated from the cell surface to the subsynaptic pool. This translocation most likely occurs via endocytosis, since it could be blocked by procedures known to interfere with internalization mediated by clathrin-coated vesicles (low temperature, hyperosmolarity, blockade of ampiphysin-dynamin interaction). Although the level of constitutive internalization of $GABA_AR$ seems to be less than AMPA receptors (Figs. 4-5), this translocation is sufficiently rapid to allow the detection of clustered, internalized receptors within about 20 min. It is therefore a major finding of the present study that constitutive endocytosis of $GABA_AR$ apparently serves to replenish the subsynaptic pool of $GABA_AR$. Furthermore, this observation might explain why, in other studies, cell-surface $GABA_AR$ labeled in living cultures at room temperature (for example, Brunig et al., 2002a; Levi et al., 2004) apparently were clustered at postsynaptic sites.

This phenomenon is specific for $GABA_AR$, since internalized AMPA receptors tagged with an antibody were not redirected to presumptive postsynaptic sites, but accumulated within the cell body. The difference is the more striking, as internalization is blocked in both cases by interference with clathrin-coated vesicle formation (Carroll et al., 1999b; Man et al., 2000). The fate and intracellular sorting of internalized receptors are therefore distinct for AMPA receptors and $GABA_AR$. A second difference stems from our observation that the internalization rate of tagged $GABA_AR$ was not influenced by exposure to THIP, bicuculline, or diazepam, whereas agonist exposure is well known to induce internalization of AMPA receptors (Carroll et al., 1999a; Beattie et al., 2000; Ehlers, 2000). A recent study has shown, however, that enhancing network activity in hippocampal slices leads to increased internalization of $GABA_AR$ (Blair et al., 2004). It is therefore possible that neuronal activity indirectly influences $GABA_AR$ internalization by activating signal transduction pathways controlled by G-protein-coupled receptors or tyrosine kinase receptors. This hypothesis is in line with the demonstration that several types of metabotropic receptors, including muscarinic acetylcholine receptors (Brandon et al., 2002), dopamine receptors (Flores-Hernandez et al., 2000), and 5-HT receptors (Huidobro-Toro et

al., 1996; Feng et al., 2001), modulate phosphorylation mechanisms that regulate the internalization of $GABA_AR$.

Little is known about intracellular trafficking of $GABA_AR$ following synthesis or internalization. A first major question raised by the present results concerns the trafficking of internalized $GABA_AR$, likely found in vesicles, towards the subsynaptic compartment. Among the $GABA_AR$-interacting factors, brefeldin A-inhibited GDP/GFP exchange factor 2 (BIG2), which binds to the cytoplasmic loop of β subunit variants, has been located in vesicles in synapses and in the dendritic cytoplasm, sometimes co-localized with $GABA_AR$ (Charych et al., 2004). BIG2 is homologous to proteins involved in vesicular trafficking and might therefore contribute to redirect internalized $GABA_AR$ to subsynaptic sites or regulate their cell-surface expression. Another potentially important factor is the ubiquitin-related protein PLIC-1, which has been suggested to stabilize intracellular $GABA_AR$ and prevent their degradation by the proteasome (Bedford et al., 2001). Interference of the interaction between PLIC-1 and native $GABA_AR$, via their α subunit variants, results in decreased cell surface expression, presumably due to enhanced degradation. Finally, palmytoylation of $GABA_AR$ has recently been shown to be essential for their postsynaptic clustering (Rathenberg et al., 2004). This posttranslational modification involves cystein residues in the third cytoplasmic loop of the γ2 subunit, which is present in $GABA_AR$ clustered at postsynaptic sites. A possible candidate mediating palmytoylation is a novel $GABA_AR$-associated membrane protein, named Golgi apparatus-specific protein with the DHHC zinc finger domain (GODZ), and which selectively associates with the γ2 subunit (Keller et al., 2004). However, since GODZ is highly enriched in the Golgi apparatus, it is not yet clear whether it interacts also with newly internalized receptors.

A second major question arises from the observation that the staining intensity of gephyrin clusters is reduced upon blockade of $GABA_AR$ internalization with the dynamin-inhibiting peptide. It has been shown previously that clustering of gephyrin depends on $GABA_AR$ clustering in both developing and mature synapses (Essrich et al., 1998; Schweizer et al., 2003). The present results suggest that the amount of gephyrin at postsynaptic sites is regulated on a short-time basis in function of the amount of $GABA_AR$. In cultures from gephyrin-deficient mice, $GABA_AR$ clustering is only partially impaired, resulting in decreased amplitude of mIPSCs compared to wild type (Levi et al., 2004). Our observations therefore raise the possibility that gephyrin plays a role for the clustering of the subsynaptic, intracellular pool of $GABA_AR$, but not of receptors inserted in the cell membrane.

The biotinylation assay revealed that the amount of internalized β3 subunit is larger than that of the β2 subunit (Fig. 5F), suggesting that endocytosis of the corresponding receptor subtypes is regulated differentially. A possible mechanism is the presence of consensus sites for phosphorylation by both PKC and PKA in the β3 subunit, whereas only PKA

phosphorylation sites are found on the β2 subunit (Kittler and Moss, 2003). In view of the differential role of these two kinases in regulating cell-surface expression of $GABA_AR$, it is therefore conceivable that phosphorylation by PKC mediates most of the constitutive internalization occurring in our assay. However, since the β2 subunit is presumably associated mainly with the α1 subunit in interneurons, its internalization should be detectable in a more sensitive assay.

Functional significance

Endocytosis of ionotropic receptors is being recognized as a major facet of short- and long-term synaptic plasticity. As shown best for AMPA receptors, it is a highly regulated mechanism, involving differential interactions with distinct receptor subtypes and with scaffolding proteins (Bredt and Nicoll, 2003). An additional component is the lateral mobility of the receptors (Borgdorff and Choquet, 2002), allowing a rapid exchange between synaptic and extrasynaptic receptors, and differential endocytosis of the two receptor populations (Ashby et al., 2004; Groc et al., 2004). Much less is known about the mobility of receptors mediating synaptic inhibition, such as glycine receptors (Meier et al., 2001) and $GABA_AR$ (Meissner and Haberlein, 2003), in spite of the fact that the latter are the target of clinically important drugs used to treat chronic disorders, such as epilepsy or anxiety. Although a rapid trafficking of cell-surface receptors has been demonstrated (Tehrani and Barnes, 1997; Wan et al., 1997), the effects of chronic drug exposure, in particular benzodiazepines, are controversial. While tolerance to the pharmacological action of these drugs does not involve major changes in $GABA_AR$ expression (van Rijnsoever et al., 2004), internalization of recombinant receptors expressed in Sf9 cells has been reported upon long-term diazepam exposure (Ali and Olsen, 2001). Increased internalization of the α1 subunit occurred only after a 7-day lorazepam treatment (Tehrani and Barnes, 1997), whereas a similar treatment with flurazepam resulted in decreased PKA activity correlating with reduced mIPSCs in slices from treated animals (Lilly et al., 2003). The possible involvement of internalization mechanisms during the development of tolerance to benzodiazepines is supported by the observation that tolerance is reversed quickly upon administration of the antagonist flumazenil, suggesting that it is based on a rapidly reversible biochemical process (Gonsalves and Gallager, 1988; Tietz et al., 1999).

However, in contrast to these speculations, the present findings of a large pool of $GABA_AR$ directly under the synaptic membrane rather suggest that $GABA_AR$ endocytosis contributes to short-term regulation of GABAergic inhibitory transmission in direct response to acute changes in network activity, thereby providing a novel mechanism of synaptic plasticity. Furthermore, the observation that $GABA_AR$ expression is upregulated in chronic diseases such as epilepsy (Loup et al., 2000; Knuesel et al., 2001) suggests that dysregulation of the trafficking mechanisms contributes to the pathophysiology of these disorders.

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3.2 Requirement of α5-GABA$_A$ receptors for normal development of tolerance to the sedative action of diazepam

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Abstract

Despite its pharmacological relevance, the mechanism of the development of tolerance to the action of benzodiazepines is largely unknown. The acute sedative action of diazepam is mediated via α1-GABA$_A$ receptors. It was therefore tested whether chronic activation of these receptors by diazepam is sufficient to induce tolerance to its sedative action. Knock-in mice, in which the α1-, α2-, α3- or α5-GABA$_A$ receptors had been rendered diazepam-insensitive by histidine/arginine point mutation, were chronically treated with diazepam (8 days, 15 mg/kg/day) and tested for motor activity. Wild type, α2(H101R), and α3(H126R) mice showed a robust diminution of the motor depressant drug action. In contrast, α5(H105R) mice failed to display any sedative tolerance. α1(H101R) mice showed no alteration of motor activity upon chronic diazepam treatment. Autoradiography with [^{3}H]flumazenil revealed no change in benzodiazepine binding sites. However, a decrease of α5-subunit radioligand binding was detected selectively in the dentate gyrus with specific ligands. This alteration was observed only in diazepam-tolerant animals, indicating that the manifestation of tolerance to the sedative action of diazepam is associated with a down regulation of α5-GABA$_A$ receptors in the dentate gyrus. Thus, the chronic activation of α5-GABA$_A$ receptors is crucial for the normal development of sedative tolerance to diazepam, which manifests itself in conjunction with α1-GABA$_A$ receptors.

Introduction

Loss of sedative efficacy of diazepam upon chronic treatment has been proposed to result from the development of adaptive processes counteracting the repeated enhancement by the benzodiazepine of γ-aminobutyric acid $(GABA)_A$ receptor-mediated inhibitory neurotransmission (Fernandes et al., 1996; Marin et al., 1996; File and Fernandes, 1994; Steppuhn and Turski, 1993; Marin et al., 1999; Perez et al., 2003). Functional alterations of $GABA_A$ receptors have frequently been reported upon various chronic treatment regimens with diazepam (Hutchinson et al., 1996; Bateson, 2002; Costa et al., 2001; Itier et al., 1996; Primus et al., 1996; Ali and Olsen, 2001). Uncoupling of the allosteric interaction between the benzodiazepine binding site and the GABA site, probably linked to $GABA_A$ receptor internalisation, has been proposed as a correlate of diazepam tolerance (Hutchinson et al., 1996; Costa et al., 2001; Ali and Olsen, 2001; Itier et al., 1996; Primus et al., 1996). Furthermore, subtle changes in the expression of $GABA_A$ receptor subunits were described notably in the cerebral cortex and hippocampus (Wu et al., 1994; Arnot et al., 2001; Impagnatiello et al., 1996; Pesold et al., 1997). In particular, a selective decrease in the expression of genes encoding the α1- and γ2-subunits in dendrites and spines of cortical pyramidal cells has been associated with tolerance to the anticonvulsant action of diazepam (Costa et al., 2002). In addition, α5-$GABA_A$ receptors were affected, as shown by an increase in α5-subunit mRNA in frontoparietal cortex or a reduced radioligand binding in hippocampus after two or three weeks of diazepam administration (Impagnatiello et al., 1996; Pesold et al., 1997; Li et al., 2000).

The recognition of distinct pharmacological functions of $GABA_A$-receptor subtypes opened new avenues to investigate the mechanisms of tolerance. Using a histidine-to-arginine point mutation strategy that selectively abolishes diazepam binding to $GABA_A$ receptors containing the α1-, α2-, α3- or α5-subunit *in vivo*, it has been shown that α1-$GABA_A$ receptors mediate the acute sedative (Rudolph et al., 1999; McKernan et al., 2000) and anticonvulsant (Rudolph et al., 1999) properties of diazepam, whereas α2-$GABA_A$ receptors are the substrate for anxiolytic (Löw et al., 2000) and muscle relaxant activity, the latter requiring also α3- and α5-$GABA_A$ receptors (Crestani et al., 2001; Crestani et al., 2002). In view of such functional receptor specificity, it was tested whether tolerance to a particular effect of diazepam is mediated via the same receptor subtype involved in the acute effect, or whether additional $GABA_A$ receptor subtypes are required for neuronal plasticity leading to the development of tolerance upon chronic diazepam treatment.

In the present study, the contribution of specific $GABA_A$-receptor subtypes in the development of tolerance to the motor depressant action of diazepam was examined using wild type and histidine/arginine point mutated mice. Potential changes in benzodiazepine-binding sites were analyzed by autoradiography.

Methods

Animals and drugs. 9- to 11-week old female wild type, α1(H101R), α2(H101R), α3(H123R), and α5(H105R) mice (> 10 backcrosses to 129/SvJ background) were used (Rudolph et al., 1999; Löw et al., 2000; Crestani et al., 2002). The histidine/arginine substitution in the $GABA_A$ receptor α1-, α2, α3- or α5-subunit results in a marked reduction of the binding affinity of the corresponding $GABA_A$ receptor subtype to diazepam, as shown on recombinant receptors (Benson et al., 1998). Thus, each point-mutated mouse line possesses a particular $GABA_A$ receptor subtype insensitive to allosteric modulation by diazepam whilst its response to GABA is largely preserved. Females were preferred to males because of the prominent intramale aggressive behaviour inherent to the strain used as genetic background. They were reared in group-housed cages in the testing room under reversed 12-hour light/dark conditions. Treatments and behavioral testing were performed during the dark phase. The Cantonal Veterinary Office of Zurich approved all experimental procedures. Diazepam was from F. Hoffmann-La Roche (Basel, Switzerland).

Induction of sedative tolerance. Mice were subjected to daily injections of diazepam (10 mg/kg at 10 am and 5 mg/kg at 4 pm; intraperitoneally) for 8 days in the home-cage. On day 9, half of the mice were given vehicle and the other half, the morning dose of 10 mg/kg of diazepam as test dose. Control animals, which received the vehicle (0.3% tween/saline solution) as chronic treatment, were distributed in two groups. One group was treated with vehicle and the other group with 10 mg/kg of diazepam. A fifth group of mice, which served as control for the effects of repeated injections, received a single diazepam (10 mg/kg) injection as test dose. Animals were left undisturbed to experience the drug effects in the home-cage for 30 min. At the end of this period, they were placed in individual circular alleys (Imetronic, Pessac, France) for motor activity assessment, measured as the number of photocell interruptions, during a 10-min period. The term “test dose” was used to indicate the association of the last diazepam morning injection of the chronic treatment with the behavioural assessment. The terms “sedation” and “sedative”, as defined in Katzung (1995), indicate the drug-induced decrease of the animal’s spontaneous activity. Measurement of motor activity in rodents represents a standard behavioral assay for testing the sedative potential of drugs (Vogel, 2002).

Quantitative receptor autoradiography. A second series of animals received the same chronic treatment regimen with either vehicle or diazepam. On day 9, tolerance to the motor depressant action of 10 mg/kg of diazepam was tested and the mice were sacrificed by decapitation 5 hours thereafter. Binding assays using [^{3}H]flumazenil (NEN, Perkin-Elmer, Boston, USA), [^{3}H]RY80 (Perkin-Elmer, Boston, USA) or [^{3}H]L655708 (Amersham Biosciences Europe GmbH, Otelfingen, Switzerland), two ligands with a preferential

affinity for the α5-subunit (Skolnick et al., 1997; Quirk et al., 1996), were performed on transverse 12-μm brain cryosections (Fritschy et al., 1997). Briefly, after a 1-hr incubation with 12 nM of [^{3}H]flumazenil or 2 nM of either [^{3}H]RY80 or [^{3}H]L655708 in 50 mM Tris/Cl at pH 7.5, the sections were exposed to a tritium sensitive phosphor screen (Packard Cyclone Storage Phosphor System) for 2 or 8 days, respectively. Adding 10 μM of clonazepam assessed unspecific labeling. The screens were digitized with a Packard Cyclone Scanner and labeling intensities were measured in motor cortex, striatum, nucleus accumbens, and hippocampal formation (CA1, CA3 stratum oriens/pyramidale and dentate gyrus) of both hemispheres. At the concentrations used, both [^{3}H]RY80 and [^{3}H]L655708 have been reported to saturate with a high affinity α5-subunit binding sites in hippocampal membranes (Skolnick et al., 1997; Quirk et al., 1996; Sur et al., 1999).

Statistics. Results, expressed as mean ± SE (or SD for binding studies), were analyzed using non-parametric Kruskall-Wallis analysis and, whenever appropriate, Mann-Whitney's tests for post-hoc mean comparisons.

Results

Tolerance to the sedative action of diazepam in point-mutated mice

To identify the diazepam-sensitive $GABA_A$ receptor subtypes implicated in sedative tolerance, we examined the potential of histidine/arginine point-mutated mice to develop tolerance against the motor depressant action of diazepam during the course of a chronic drug treatment regimen. We focused on this behavioral effect for two main reasons. Firstly, the effectiveness of diazepam in decreasing motor activity in rodents is considered as a valid behavioral manifestation of its sedative properties (Vogel, 2002). Secondly, this drug effect is exclusively mediated by α1-$GABA_A$ receptors, since it is abolished in α1(H101R) mice whilst unaltered in α2(H101R), α3(H126R) and α5(H105R) mice (Rudolph et al., 1999; McKernan et al., 2000; Löw et al., 2000; Crestani et al., 2002).

Sedative tolerance to diazepam, i.e. the diminution of its motor depressant action upon chronic treatment, was first analyzed using wild type and α1(H101R) mice. The different treatment conditions affected differently motor activity in wild type mice [$H = 22.958$, $p < 0.001$, $n = 6$ to 7 mice per group]. In wild type mice chronically treated with vehicle, the administration of 10 mg/kg of diazepam was followed by a marked decrease in motor activity ($p < 0.01$ as compared with Veh-Veh) (Fig.1 a). This effect was comparable to that seen in mice, which received a single acute injection of diazepam. Mice chronically treated with diazepam did not show a reduction in motor activity in response to the test dose of diazepam (Diaz-Diaz) and were indistinguishable from mice challenged with vehicle (Diaz-Veh) or from mice chronically treated with vehicle only (Veh-Veh) (Fig. 1 a). In

α1(H101R) mice, neither a single acute injection of diazepam (10 mg/kg) nor the chronic diazepam treatment altered the level of motor activity in comparison to the chronic vehicle treatment, as revealed by the lack of statistical significance of the overall analysis of the effects of the different drug treatment conditions [$H = 5.914$, *not significant*, $n = 6$ mice per group] (Fig. 1 b). The increased motor activity seen in chronically vehicle-treated α1(H101R) mice in response to diazepam achieved significance only when compared, in a separate two-mean comparison, to the effect of the vehicle test injection ($p < 0.01$, Mann-Whitney) but not when compared to that induced by a single acute diazepam injection (Fig. 1 b).

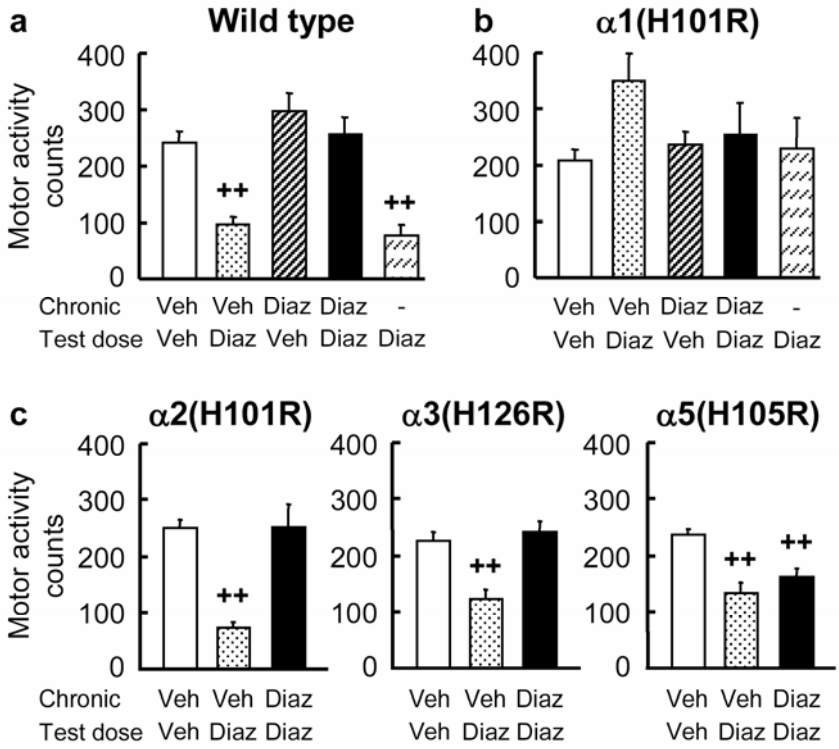

Fig. 1. Motor activity in wild type and point mutated mice subjected to an 8-day chronic diazepam treatment regimen (15 mg/kg/day). (a) In wild type mice, the test dose of diazepam (10 mg/kg) was equally effective in decreasing motor activity, when given either acutely or 18 hours after a chronic vehicle treatment. Mice chronically treated with diazepam showed levels of motor activity similar to that of mice chronically treated with vehicle, in response to either diazepam or vehicle [$H = 22.958$, $p < 0.001$, $n = 6$ to 7 mice per group]. (b) In α1(H101R) mice, there was no overall effect of the different treatment conditions on motor activity [$H = 5.914$, *not significant*, $n = 6$ mice per group]. However, an increased motor activity was seen in animals chronically treated with vehicle in response to diazepam ($p < 0.01$ as compared with Veh-Veh, Mann-Whitney). (c) The test dose of diazepam decreased motor activity in animals chronically treated with the vehicle but not in those treated with diazepam in α2(H101R) [$H = 14.942$, $p < 0.001$, $n = 8$ mice per group] and α3(H126R) mice [$H = 12.194$, $p < 0.002$, $n = 8$ mice per group]. In α5(H105R) mice, the same diazepam test dose depressed motor activity in animals chronically treated with either vehicle or diazepam [$H = 12.005$, $p < 0.002$, $n = 8$ mice per group]. Results are given as means ± SE. ++, $p < 0.01$ versus Veh-Veh, Mann-Whitney. Veh, vehicle; Diaz, diazepam.

To characterize the role of diazepam-sensitive $GABA_A$-receptor subtypes other than those containing the α1-subunit in sedative tolerance, α2(H101R), α3(H126R), and α5(H105R) mice were subjected to the same chronic diazepam treatment regimen. There was a

significant overall effect of the different treatment conditions on motor activity in all three mutant lines [α2(H101R): $H = 14.942$, $p < 0.001$, $n = 8$ mice per group; α3(H126R) mice: $H = 12.194$, $p < 0.002$, $n = 8$ mice per group and α5(H105R) mice: $H = 12.005$, $p < 0.002$, $n = 8$ mice per group]. Diazepam (10 mg/kg) failed to decrease motor activity levels in α2(H101R) and α3(H126R) mice chronically treated with diazepam while it induced sedation in those mutants chronically treated with vehicle ($p < 0.01$ as compared with the respective Veh-Veh groups) (Fig. 1 c). In contrast, in α5(H105R) mice, the same test dose of diazepam was equally effective in depressing motor activity in animals chronically treated with either vehicle or diazepam ($p < 0.01$ as compared with Veh-Veh) (Fig. 1 c). Thus, tolerance to the motor depressant action of diazepam developed to the same extent in wild type, α2(H101R) and α3(H126R) mice within 8 days of chronic drug administration, whilst it failed in α5(H105R) mice; furthermore, the same chronic diazepam treatment regimen did not alter motor activity in α1(H101R) mice.

Brain autoradiography of α5-$GABA_A$ receptor-binding sites

To assess whether the expression of sedative tolerance to diazepam would be associated with alterations in $GABA_A$ receptor binding sites, we analyzed benzodiazepine binding autoradiographically with [^{3}H]flumazenil in wild type, α1(H101R) and α2(H101R) mice. The levels of [^{3}H]flumazenil binding differed across genotypes due to the presence of the respective point mutation (Fig. 2). However, no alteration in relation with the chronic drug treatment regimen and the behavioral testing was detected, in particular in forebrain regions involved in the control of motor activity, including primary motor cortex, striatum, and nucleus accumbens, as well as in the hippocampal formation (Fig. 2). Thus, there was no evidence for a general $GABA_A$ receptor down-regulation.

To specifically analyze the role of α5-$GABA_A$ receptors, two selective α5-subunit ligands, [^{3}H]RY80 and [^{3}H]L655708, were used in the autoradiographic analysis. In wild type mice, the levels of [^{3}H]RY80 binding were differentially affected by the three treatment conditions in the dentate gyrus [$H = 14.769$, $p < 0.001$, $n = 7$ to 8 mice per group] (Fig. 3 a). A significantly lower [^{3}H]RY80 binding level (-13.6% relative to Veh-Veh) was observed in diazepam-tolerant animals ($p < 0.01$ as compared with Veh-Veh and Veh-Diaz). A similar effect of the chronic diazepam treatment was confirmed using [^{3}H]L655708 as radioligand [$H = 10.903$, $p < 0.004$, $n = 6$ to 7 mice per group] (Fig. 3 b). The [^{3}H]L655708 binding level was significantly reduced (14.7% relative to Veh-Veh) only in tolerant mice ($p < 0.01$ as compared with Veh-Veh and Veh-Diaz).

Chronic treatment Test dose	Wild type Veh Veh	 Veh Diaz	 Diaz Diaz	α1(H101R) Veh Veh	 Veh Diaz	 Diaz Diaz	α2(H101R) Veh Veh	 Veh Diaz	 Diaz Diaz
Motor cortex	183.8±4.8	182.0±2.9	199.6±11.3	61.3±2.0	64.1±1.0	56.3±2.1	154.2±2.8	155.2±5.1	152.3±4.2
Striatum	74.1±2.9	72.0±3.1	76.0±5.4	43.8±1.9	43.5±1.9	39.4±1.0	47.6±1.7	45.3±1.7	43.9±1.6
N.Accumbens	61.4±2.5	61.2±1.6	59.1±4.3	68.6±1.7	72.4±5.1	66.3±1.8	51.2±2.8	47.9±2.0	46.1±2.2
Hippocampus	150.1±5.5	142.0±7.4	140.8±9.2	94.7±4.6	95.1±6.0	88.4±4.1	110.9±12.1	110.5±10.0	115.1±8.3

Fig. 2. Autoradiography of [^{3}H]flumazenil binding after cessation of an 8-day treatment regimen with vehicle or diazepam (15 mg/kg/day) and administration of the test dose diazepam (10 mg/kg) in wild type, α1(H101R), and α2(H101R) mice. Irrespective of the genotype, the chronic diazepam treatment did not alter [^{3}H]flumazenil binding compared to chronic vehicle or acute diazepam, as quantified for three regions involved in motor control and for the hippocampal formation. Standards and differential [^{3}H]flumazenil binding profile related to the point-mutation are also presented in representative transverse sections from chronically vehicle-treated animals. The different binding levels in the mutant mice reflect the loss of diazepam binding to the mutated subunit. Results are expressed in mean nCi/mg protein ± SD, n = 4 – 7 mice per group. Veh, vehicle; Diaz, diazepam.

In contrast, the effects of the three treatment conditions on [^{3}H]RY80 and [^{3}H]L655708 binding levels were comparable in the hippocampal CA1 area [[^{3}H]RY80: H = 1.563, not significant, n = 5 to 6 mice per group and [^{3}H]L655708: H = 3.661, not significant, n = 6 to 7 mice per group] as well as in the CA3 area [[^{3}H]RY80: H = 4.662, not significant, n = 5 to 6 mice per group and [^{3}H]L655708: H = 1.988, not significant, n = 6 to 7 mice per group] (not shown).

[^{3}H]RY80 binding was further analyzed in α1(H101R) and α2(H101R) mice subjected to the same chronic drug treatment and behavioral testing. In α2(H101R) mice, as in wild type mice, the chronic administration of diazepam was accompanied by sedative tolerance (not shown) and by a decrease of [^{3}H]RY80 binding level in dentate gyrus (-12.5 % relative to Veh-Veh, $p < 0.01$) [H = 9.231, $p < 0.009$, n = 5 to 6 mice per group] (Fig. 3 c). The α1(H101R) mice displayed similar levels of [^{3}H]RY80 binding, irrespective of the treatment conditions [H = 2.788, not significant, n = 5 to 6 mice per group] (Fig. 3 d). Thus, the manifestation of tolerance to the motor depressant action of diazepam was accompanied by an apparent reduction of α5-$GABA_A$ receptor binding selectively in the dentate gyrus, as shown for wild type and α2(H101R) mice. This reduction of α5-subunit binding did not occur in α1(H101R) mice subjected to the same chronic diazepam treatment.

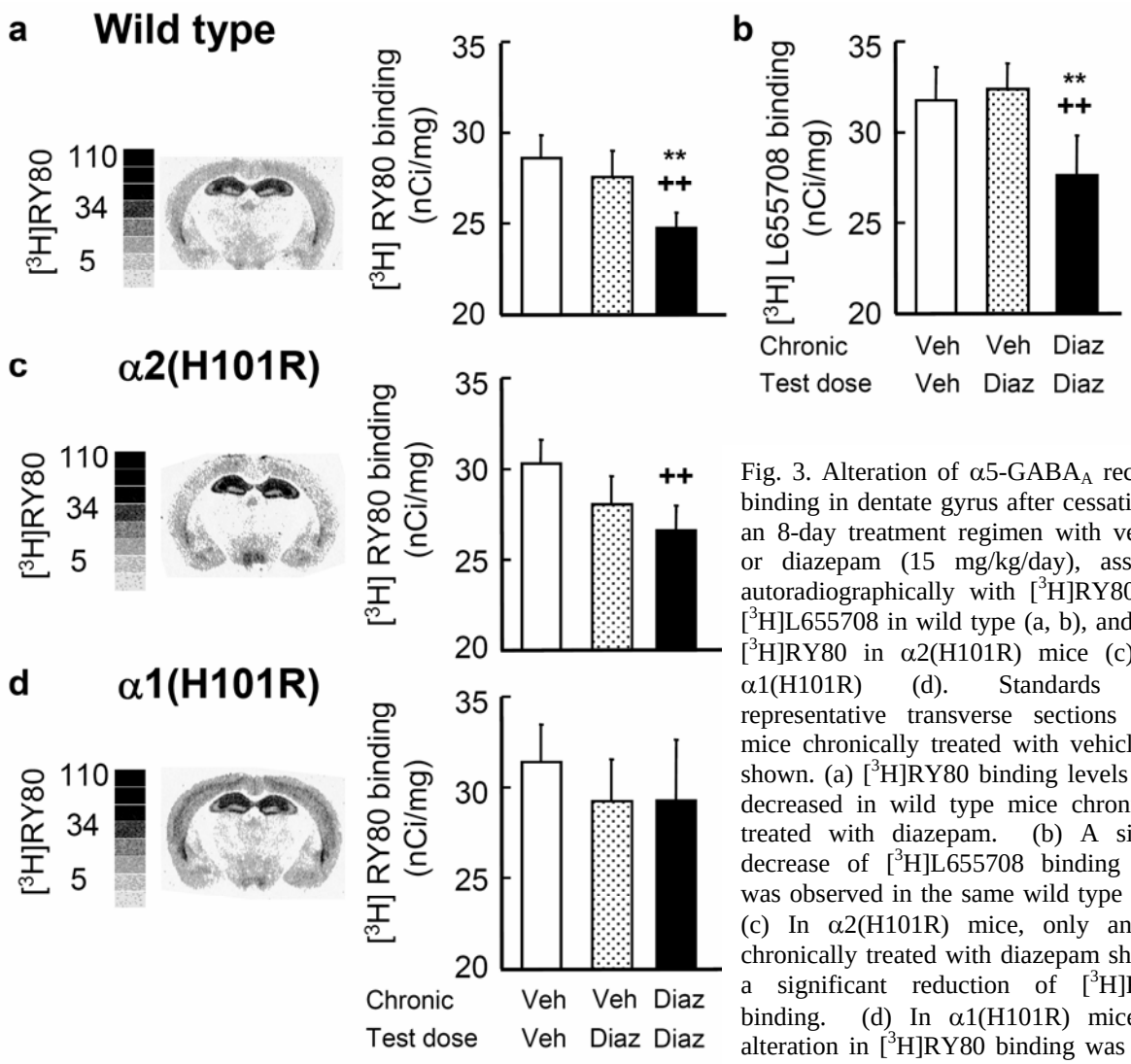

Fig. 3. Alteration of α5-$GABA_A$ receptor binding in dentate gyrus after cessation of an 8-day treatment regimen with vehicle or diazepam (15 mg/kg/day), assessed autoradiographically with [^{3}H]RY80 and [^{3}H]L655708 in wild type (a, b), and with [^{3}H]RY80 in α2(H101R) mice (c) and α1(H101R) (d). Standards and representative transverse sections from mice chronically treated with vehicle are shown. (a) [^{3}H]RY80 binding levels were decreased in wild type mice chronically treated with diazepam. (b) A similar decrease of [^{3}H]L655708 binding level was observed in the same wild type mice. (c) In α2(H101R) mice, only animals chronically treated with diazepam showed a significant reduction of [^{3}H]RY80 binding. (d) In α1(H101R) mice, no alteration in [^{3}H]RY80 binding was seen, irrespective of the chronic drug treatment. Results are given as mean nCi/mg protein ± SD. ++, $p < 0.01$ versus Veh-Veh, **, $p < 0.01$ versus Veh-Diaz, Mann-Whitney test. Veh, vehicle; Diaz, diazepam

Discussion

The present results point to a critical role of α5-$GABA_A$ receptors, in conjunction with α1-$GABA_A$ receptors, for the development of tolerance against the sedative action of diazepam. Interaction of diazepam with α5-$GABA_A$ receptors appears to be a prerequisite for the normal development of tolerance against its motor depressant action. This is shown by the retained capacity of diazepam (10 mg/kg) to reduce efficiently motor activity in α5(H105R) mice, which possess diazepam-insensitive α5-$GABA_A$ receptors, at least within the limits of our chronic drug treatment regimen (Fig. 1 c). This motor effect cannot be attributed to a retained myorelaxant action of diazepam, since we have shown that these mutants, as well as the α2(H101R) mice, do not express the acute myorelaxant action of diazepam in the range of doses used for the chronic treatment (Crestani et al., 2001; Crestani et al., 2002).

On the other hand, α2(H101R) and α3(H126R) mice developed sedative tolerance to diazepam to the same extent as wild type mice (Fig. 1 c), indicating that diazepam-sensitive α2- or α3-$GABA_A$ receptors are not essential for this effect. In rodents, tolerance to the motor depressant action of diazepam develops rapidly, within 3 to 5 days of chronic administration (Marin et al., 1996; File & Fernandes, 1994; Steppuhn & Turski, 1993). We designed a chronic treatment protocol, using high doses of diazepam over a period of 9 days, in order to induce a robust sedative tolerance in our control wild type mice. Nevertheless, we cannot rule out the possibility of a delayed occurrence of sedative tolerance, i.e. upon a longer chronic diazepam treatment, in α5(H105R) mice. Conversely, the absence of motor depressant drug effect in wild type mice, α2(H101R) and α3(H126R) mice upon chronic treatment could reflect a rightward shift in the dose-response curve of diazepam, so that the same chronically diazepam-treated animals would show sedation in response to higher doses of diazepam (>10 mg/kg). However, this appears improbable, especially with regard to our test conditions. Motor activity was measured 30 mn after the last diazepam injection of the chronic treatment, and not after a period of drug withdrawal as this is often the case in the literature, in order to assess the retained sedative drug efficacy at a particular time of the chronic diazepam treatment regimen. To our knowledge, only the study of Perrault et al. (1993), using the anticonvulsant action of diazepam as endpoint, described a rightward and downward shift in the dose-response curve of diazepam, but only when the test dose was given 42 hours after termination of a 10-day chronic treatment regimen (2 x 5 mg/kg po). This shift was attributed to the rapid development of a hypersensitivity of the mice to the convulsant drug, in relation to diazepam withdrawal experience. A significant residual anticonvulsant action of diazepam was seen in chronically treated animals when the convulsant drug was administered 6 hours after the last diazepam dose (Perrault et al., 1993).

The diminution of motor activity seen in α5(H105R) mice upon chronic diazepam treatment further reveals that, during the course of the chronic diazepam treatment, α1-$GABA_A$ receptors remain responsive, mediating recurrently sedation, in these mutants. Therefore, sedative tolerance appears not to be due to a reduction of the motor depressant efficacy of diazepam, thus confirming a previous report (Bourin et al., 1992). Rather, its manifestation might be secondary to the development of an α5-$GABA_A$ receptor-dependent response to chronic diazepam, which would oppose its motor depressant action. In α1(H101R) mice, which do not display the sedative drug action, chronic interaction of diazepam with $GABA_A$ receptors other than those containing the α1 subunit was not associated with change in motor activity (Fig. 1 b). This result argues against an oppositional α1-$GABA_A$ receptor-independent mechanism, which would counterbalance, thus masking, the sedative drug action. α1- and α5-$GABA_A$ receptors appear to be the specific molecular substrates

contributing in a competitive manner to the chronic effects of diazepam on motor activity. Chronic interaction of diazepam with α1-$GABA_A$ receptors results in a recurrent motor depressant action while the concurrent interaction with α5-$GABA_A$ receptors is essential for the behavioral manifestation of tolerance to this effect. This result is in line with reports that benzodiazepine site ligands that do not interact with α5-$GABA_A$ receptors, such as zolpidem, show little or no evidence for sedative tolerance in rodents and fail to alter α5-subunit levels (Zivkovic et al., 1994; Costa and Guidotti, 1996; Holt et al., 1997).

The α5-$GABA_A$ receptors of the dentate gyrus appear to be a specific target for adaptive changes associated with sedative tolerance to diazepam. Indeed, a down regulation of α5-$GABA_A$ receptors, as assessed with two selective α5-subunit radioligands used at saturating concentrations, was observed only in wild type and α2(H101R) mice tolerant to the motor depressant action of diazepam (Fig. 3 a, b, and c). This reduction of receptors was restricted to the dentate gyrus. No change in α5-subunit radioligand binding levels was detected in the hippocampal CA1 and CA3 area of these animals. Within the limits of statistical power, a decreased binding of α5-subunit-specific radioligands in diazepam-tolerant animals could be resolved only in the hippocampal formation. We do not exclude that the chronic diazepam treatment regimen used here can give rise to alterations in α5-$GABA_A$ receptors in other brain regions. However, the unchanged levels of [^{3}H]flumazenil binding in forebrain regions involved in control of motor activity argues against a general down-regulation of $GABA_A$ receptors, as well as against a regionally-specific loss of another major $GABA_A$ receptor subtype after chronic diazepam treatment. This observation is in concordance with many other reports that flumazenil binding sites remain unaltered upon chronic benzodiazepine treatment (Hutchinson et al., 1996; Bateson, 2002; Costa et al., 2001). The absence of alteration in α5-subunit radioligand binding in α1(H101R) mice chronically treated with diazepam (Fig. 3 d) further indicates that occurrence of the α5-$GABA_A$ receptor down-regulation in the dentate gyrus closely depends on the chronic interaction of the drug with α1-$GABA_A$ receptors but not with $GABA_A$ receptors containing the α2-, α3- or α5-subunit.

α5-$GABA_A$ receptors constitute a minor population of diazepam-sensitive $GABA_A$ receptors. They are found mainly in the hippocampal formation, olfactory bulb granule cell layer, and spinal cord dorsal horn, and in lower amounts, in the cerebral cortex and hypothalamus (Crestani et al., 2002). We have previously reported that the H105R point mutation in the α5 subunit gene resulted in a reduction of α5-$GABA_A$ receptors in the dendritic layers of the hippocampal CA1 and CA3 areas with no change in the dentate gyrus (Crestani et al., 2002). Although this specific deficit of receptors in α5(H105R) mice mimics to some extent the molecular changes seen in the dentate gyrus of diazepam-tolerant

animals, it does not interfere with the expression of the motor depressant action of diazepam, whenever given acutely (Crestani et al., 2002) or chronically (Fig. 1 c). This is in keeping with the lack of alteration in the expression pattern of the other major $GABA_A$ receptor subunits, notably the α1-subunit, in α5(H105R) mice (Crestani et al., 2002) as well as in mice with a complete loss of hippocampal α5-$GABA_A$ receptors (Collinson et al., 2002). Thus, it is not a reduction in the expression of α5-$GABA_A$ receptors *per se*, but rather its selective localization in the dentate gyrus, which appears to be associated with the expression of sedative tolerance to diazepam. Likewise, the induction of long-term potentiation in hippocampal pyramidal cells is unaltered in α5(H105R) mice (Crestani et al., 2002) whereas tolerance to the motor depressant action of diazepam is associated with an increased synaptic plasticity in the rat dentate gyrus (Marin et al., 1996). Therefore, the failure of α5(H105R) mice to manifest sedative tolerance, despite their partial deficit in hippocampal α5-$GABA_A$ receptors, strengthens the hypothesis that diazepam binding to α5-$GABA_A$ receptors is a key mechanism underlying the robust diminution of its sedative efficacy upon chronic treatment. This is the more striking, as these receptors are mainly extrasynaptic and mediate tonic inhibition in hippocampal CA1 pyramidal cells (Crestani et al., 2002; Caraiscos et al., 2004). The association of changes in α5-subunit binding sites, mRNA, or protein levels with tolerance to diazepam has been reported previously for its anticonvulsant properties (Li et al., 2000; Pesold et al., 1997; Impagnatiello et al., 1996; Wu et al., 1994). Here, we demonstrate that the decrease in α5-subunit binding in the dentate gyrus depends on the chronic activation by diazepam of α1-$GABA_A$ receptors, which primarily produce phasic inhibition in the brain. This is in keeping with the reported high plasticity in the expression of hippocampal extrasynaptic α5-$GABA_A$ receptors in response to intense synaptic activity (Houser and Esclapez, 2003).

In conclusion, we propose that the manifestation of tolerance to the motor depressant action of diazepam depends on the chronic activation of two competitive mechanisms orchestrated by respectively α1- and α5-$GABA_A$ receptors. 1) Chronic drug interaction with α1-$GABA_A$ receptors results in a persistent augmentation of the phasic inhibition in the forebrain areas involved in motor control, mediating motor depression. This recurrent increased phasic signaling would alter the weight of the tonic inhibition produced by 2) the simultaneous drug activation of extrasynaptic α5-$GABA_A$ receptors in the hippocampal formation. The α1-$GABA_A$ receptor-dependent changes in inhibitory efficacy, which occurs during the course of the chronic diazepam treatment, is reflected by the 15% diminution of α5-$GABA_A$ receptors in the dentate gyrus of tolerant animals. This is in keeping with the potential of a small reduction in the efficacy of $GABA_A$ receptor-mediated inhibition (~10%) to markedly increase cortical excitation (Chagnac-Amitai and Connors, 1989). Tolerance against the motor depressant action of diazepam has been associated with an enhancement of both

hippocampal synaptic efficacy and NMDA receptor subunit mRNAs expression in dentate gyrus (Marin et al., 1996; Perez et al., 2003). Moreover, it has been reported that acute application of diazepam on hippocampal slices prevents the long-term potentiation of population spikes, whereas zolpidem has no effect, suggesting a possible α5-$GABA_A$ receptor-dependent mechanism (Higashima et al., 1998). We do not exclude a compensatory alteration in diazepam-insensitive tonic inhibition mediated by δ-subunit containing $GABA_A$ receptors in the dentate granule cells, which could contribute to the increase in hippocampal excitability upon chronic diazepam treatment (Nusser and Mody, 2002). However, the high relative abundance of diazepam-sensitive α5-$GABA_A$ receptors in the hippocampal formation positions this structure as a key player for development of the sedative tolerance phenotype by means of its widespread efferent connections to brain areas involved in the regulation of motor control.

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3.3 Selective enhancement of α1-$GABA_A$ receptor activity by zolpidem is insufficient to induce full sedative tolerance and hippocampal α5-$GABA_A$R downregulation.

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In preparation.

Introduction

The tolerance liability of benzodiazepine site ligands depends on their efficacy (Johnston and Bristow, 1998) and subtype selectivity (Bateson, 2002). Full agonists that bind to all diazepam-sensitive $GABA_A$ receptor ($GABA_AR$) subtypes with the same affinity are more prone to the development of tolerance then partial agonist such as imidazenil (Auta et al., 1994). In recent years, subtype specific ligands, such as zolpidem, were developed that showed only limited tolerance to their effects in rodents and in clinical practice (Rush, 1998). Zolpidem is an imidazopyridine with a high affinity for α1-containing $GABA_AR$, an intermediate affinity for α2- and α3-, and a very low affinity for α5-$GABA_AR$ (Sanna et al., 2002). Our previous study demonstrated that induction of sedative tolerance to diazepam requires chronic activation of both α1- and α5-$GABA_AR$ subtypes and is associated with decreased α5 binding sites in the dentate gyrus (van Rijnsoever 2004, previous chapter). Most importantly, the lack of sedative tolerance of α5(H101R) mice implies that α1-$GABA_AR$ remain pharmacologically responsive during the course of the chronic diazepam treatment. This suggests that the low tolerance to zolpidem is perhaps due to lack of enhancement of tonic inhibition mediated by α5-$GABA_AR$. Consequently one would also expect that changes in α5 binding sites associated with diazepam tolerance would not be detected after chronic zolpidem treatment. However, because of the point mutation, it could not be assessed in α5(H101R) whether any change occurs in α5 binding sites. As we have shown that the α2- and the α3-$GABA_AR$ subtypes are redundant for the development of sedative tolerance, zolpidem, with its high affinity for α1 subunit, should be able to mimic the situation seen in diazepam-treated α5(H101R) mice. We examined the ability of chronic zolpidem treatment to induce tolerance to its sedative action and to induce changes in the number of α5-$GABA_AR$ binding sites in wild type mice. The effects of an acute sedative dose of zolpidem on the molecular changes associated with sedative tolerance to diazepam were tested as well.

Materials & Methods

Animals and drugs. 9- to 11-week old female wild type mice (129/SvJ and NMRI background) were used. They were reared in group-housed cages in the testing room under reversed 12-hour light/dark conditions. Treatments and behavioral testing were performed during the dark phase. The Cantonal Veterinary Office of Zürich approved all experimental procedures. Diazepam was from F. Hoffmann-La Roche (Basel, Switzerland); Zolpidem from Sanofi-Synthelabo Recherches (Chilly Mazarin, France). [^{3}H]L655708 was from Amersham (Amersham Biosciences Europe GmbH, Otelfingen, Switzerland).

Chronic drug treatment. Mice were subjected to daily intraperitoneal injections of diazepam or zolpidem (10 mg/kg at 10 am and 5 mg/kg at 4 pm for 8 days in their home-cage; the dose for zolpidem was adapted from the sedative ED50 found by Sanger et al. (1996). On the 9th day, i.e. 18 hours after the last injection, the mice received a test drug dose: Vehicle treated mice received vehicle, diazepam (10 mg/kg) or zolpidem (10 mg/kg). Diazepam treated mice received a test dose of diazepam (10 mg/kg) or zolpidem (10 mg/kg). Mice chronically treated with zolpidem received a test dose of zolpidem (10 mg/kg). They were left undisturbed to experience the drug effects in the home-cage for 30 min. At the end of this period, sedative tolerance was tested behaviorally by assessing motor activity measured as the number of photocell interruptions in individual circular alleys (Imetronic, Pessac, France) for 10 min. The mice were then returned to their home-cage and were sacrificed 5 hours later for autoradiography.

To examine the time course for drug effects on motor activity, a separate group of NMRI mice (Harlan Netherlands, Horst, the Netherlands) was treated for 8 days with vehicle, diazepam or zolpidem, as described above. On day 9, they were placed in individual circular alleys for measuring motor activity for 1 hour, starting immediately after injection of the test dose. The NMRI strain is an inbred strain, classically used in behavioral pharmacology for assessing motor drug effects (Wolffgramm et al., 1994; Gaddnas et al., 2001; Millan et al., 2003).

Autoradiography. Binding assays using [^{3}H]L655708, a radioligand with a preferential affinity for the α5-subunit, were performed on transverse 12-μm brain cryosections. Briefly, after a 1-hr incubation with a saturating dose (2 nM) [^{3}H]L655708 in 50 mM Tris/Cl at pH 7.5, the sections were exposed to a tritium sensitive phosphor screen (Packard Cyclone Storage Phosphor System) for 8 days. Adding 10 μM of clonazepam assessed unspecific labeling. The screens were digitized with a Packard Cyclone Scanner and labeling intensities were measured in the hippocampal formation (CA1, CA3 stratum oriens/pyramidale and dentate gyrus) of both hemispheres.

Statistics. Results, expressed as mean ± SE, were analyzed using non-parametric Kruskal-Wallis analysis and, whenever appropriate, Mann-Whitney's tests for mean comparisons. For the time course, a two-way ANOVA was conducted on the first 40 min. of recording followed by a Least Significant Difference test for mean comparisons.

Results

Partial tolerance to the sedative action of zolpidem with chronic treatment

We tested whether a chronic treatment with zolpidem led to tolerance against its sedative action in our wild type mice. Zolpidem, given at the dose of 10 mg/kg after the chronic vehicle treatment, induced a marked reduction in motor activity (Fig 1a, $P<0.01$ compared to Veh-Veh). The same test dose of zolpidem was effective in depressing motor activity ($P<0.01$, compared to Veh-Veh) but to a much lesser extent in mice chronically treated with zolpidem (Fig. 1a; $P<0.01$, compared to Veh-Zolp) [H 17.90, $P<0.001$, n= 7 to 8 mice per group]. For comparison, full tolerance to the sedative effect of diazepam developed with chronic administration (Fig 1b; $P<0.05$ compared to Veh-Diaz).

To test if the lack of motor depression seen in chronically diazepam-treated mice after administration of the test dose of diazepam reflected an absolute loss of α1-$GABA_A$ receptor-mediated responsiveness, we challenged diazepam-tolerant animals with 10 mg/kg of zolpidem. In response to zolpidem, diazepam-tolerant mice displayed a reduction in motor activity (Fig 1b; $P<0.05$, compared to Diaz-Diaz; $P<0.05$, compared to Veh-Veh), which was similar to that seen in chronically vehicle-treated mice after acute administration of diazepam [H=13.70, $P<0.01$, n= 7 to 8 mice per group] (Fig 1b). The sedative action of zolpidem observed in diazepam-tolerant wild type mice mimicked the retained sedative action of diazepam in α5(H105R) mice with chronic diazepam treatment (Fig 1c; from van Rijnsoever et al 2004). Indeed, these mutant mice, in which the same $GABA_AR$ subtypes are stimulated by diazepam as in zolpidem-treated wild type mice, displayed a decreased motor activity with both acute and chronic diazepam treatment and thus failed to develop sedative tolerance to diazepam (Fig 1c; $P<0.01$ versus Veh-Veh) [H=12.009, $P<0.002$, n= 8 mice per group].

No change in α5 binding sites after acute or chronic zolpidem administration

[^{3}H]L655708 autoradiography was performed on the wild type animals used in the behavioral experiment described above. As previously shown, chronic, but not acute, administration of diazepam was accompanied by a reduction of α5 binding sites in the dentate gyrus from wild type mice (Fig 2; $P<0.01$, compared to Veh-Veh and Veh-Diaz). In mice chronically treated with vehicle, zolpidem or diazepam and challenged with the test dose of zolpidem, [^{3}H]L655708 levels were comparable to those of mice chronically treated with vehicle only [$F_{5, 37}$=5.127, $P<0.001$, n = 6 to 8 mice per group] (Fig. 2).

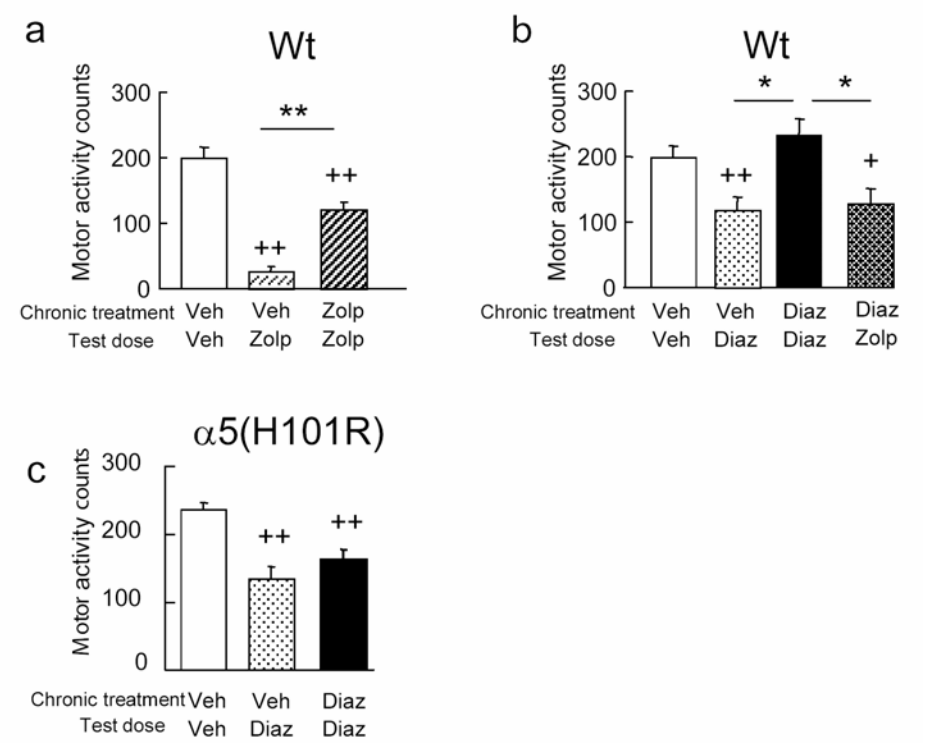

Fig 1. Effects of a test dose of zolpidem (10 mg/kg) or diazepam (10mg/kg) on motor activity after an 8-day treatment regimen with vehicle, zolpidem (15mg/kg/day) (**a**), or diazepam (15mg/kg/day) (**b** and **c**). (**a**) A test dose of zolpidem reduced motor activity in wild type mice chronically treated with vehicle, whereas this sedative effect was significantly attenuated after an 8-day zolpidem treatment regimen [H=17.90, p<0.001, n=7 to 8 mice per group]. (**b**) A test dose of diazepam depressed motor activity in vehicle-treated, but not diazepam-treated wild type mice. However, zolpidem (10mg/kg) was capable of reducing motor activity in mice that had received chronic diazepam [H=13.70, p<0.01, n=7 to 8 mice per group]. (**c**) α5(H101R) mice displayed reduced motor activity after acute and chronic diazepam treatment [H=12.005, p<0.002, n=8 mice per group, from van Rijnsoever et al. 2004]. Results are given as mean ± SE; + p<0.05, ++ p<0.01 versus Veh-Veh; ** p<0.01 versus Veh-Zolp; * p<0.05 versus Veh-Diaz; * p<0.05 versus Diaz-Diaz. Mann Whitney tests. Veh, vehicle; Zolp, zolpidem; Diaz, diazepam.

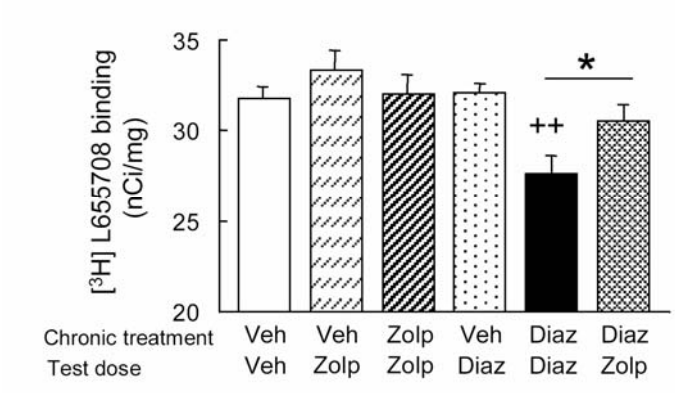

Fig 2. Levels of [^{3}H] L-655708 in dentate gyrus the day following cessation of a 8-day treatment with zolpidem (15 mg/kg/day), diazepam (15 mg/kg/day) or vehicle. Mice which were chronically or acutely treated with zolpidem showed no reduction in [^{3}H]L655708 binding in dentate gyrus 5 hours after injection of the test dose of zolpidem. Diazepam-treated mice displayed a decreased level of [^{3}H]L655708 binding in the dentate gyrus as shown previously after a test dose of diazepam (++, $P < 0.01$ compared with Veh/Veh and Veh/Diaz) but not after the test dose of zolpidem (*, $P < 0.05$ compared with Diaz/Diaz) [$F_{5, 37}$=5.127, $P <$ 0.001, n = 6 to 8 mice per group]. Results are given as means ± SE.Veh, vehicle; Diaz, diazepam; Zolp, zolpidem.

It is noteworthy that in diazepam-tolerant mice, the sedative zolpidem treatment was not associated with a downregulation of α5 binding sites (P<0.05 as compared to Diaz-Diaz, Fig. 2). No differences were observed in CA3 or CA1 (not shown). In conclusion, acute or chronic treatment with zolpidem has no effect on the expression of α5 binding sites in the dentate gyrus, even in diazepam-tolerant animals.

Sedation in chronically zolpidem treated mice immediately after injection

Although a partial tolerance to the motor depressant action of zolpidem was detected 30 min after the last injection, it was observed that animals chronically treated with zolpidem displayed sedation within minutes following zolpidem injection. Therefore, in a second experiment with NMRI mice, the effect of chronic zolpidem administration on motor activity was measured immediately after the test dose with zolpidem (10 mg/kg). Two other groups, treated chronically and tested with vehicle or diazepam in the same conditions, were used for comparison. In vehicle treated mice, motor activity is generally high in the first 5 minutes, reflecting the behavioral arousal induced by the stress and the novelty of the experimental situation. This gradually decreases over time and stabilizes to a certain level with habituation. The time course of motor activity was different in mice chronically treated with zolpidem compared to vehicle treated controls (Treatment $F_{1,7}$= 38.60, P<0.001, Time $F_{1,7}$= 23.70, P<0.001, Treatment x Time $F_{1,7,}$= 6.780, P<0.001). Zolpidem was inactive during the first 5 min following injection, as shown by the similar amount of motor activity in zolpidem and vehicle treated animals. However, from the next 5 min and for 15 min, zolpidem suppressed motor activity (10-15 min. P< 0.01; 20 min. P<0.05; compared to vehicle treated controls, Fig 3). This effect slowly disappeared 25 min after injection (Fig 3). The time course of motor activity under chronic diazepam treatment did not differ from that seen in vehicle treated animals (Treatment $F_{1,7}$= 3.596, not significant, Time $F_{1,7}$= 22.424, P<0.001, Treatment x Time $F_{1,7}$= 1.446, not significant) (Fig. 3).

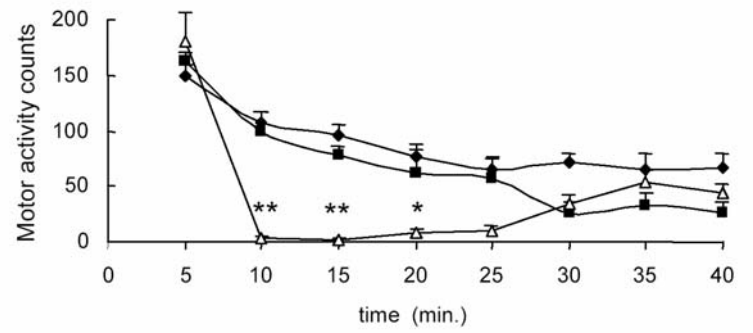

Fig 3. Time course of motor activity in mice chronically treated and tested with vehicle (♦), diazepam (■) or zolpidem (△) for 8 days. Motor activity was measured immediately after the last drug injection for 1 hour. Only the first 40 min. are shown. Note the marked motor depressant effect of zolpidem during the firts10 to 25 min. Post-injection, which gradually declines over time and the absence of difference between diazepam and vehicle-treated animals (*p<0.05, **p<0.01, compared to vehicle; Least Significant Difference test).

Thus, following 8 days of chronic administration, zolpidem was effective in suppressing motor activity during the first 20-25 min following the last injection whereas diazepam was not effective at all.

Discussion

In 129/SvJ mice, an 8-day chronic treatment with zolpidem is associated with a decrease in its motor depressant action, as tested 30 min after the last injection, which suggests a partial development of sedative tolerance. However, the analysis of the time course of the sedative effect of zolpidem after the same chronic drug treatment regimen, using NMRI mice, reveals a potent motor depressant drug effect during the first 20 min following the last injection (10 mg/kg), which gradually disappears after 30 min. For comparison, in both 129/SvJ wild type and NMRI mice, a single injection of 10 mg/kg of zolpidem results in a marked decrease in motor activity for at least 1 hour (data not shown & personal communication from F. Crestani). In contrast, the chronic administration of diazepam gives rise to the development of a robust sedative tolerance, as demonstrated by the lack of changes in motor activity following the last injection. Our results suggest that the potential of zolpidem to produce sedative tolerance with chronic administration is only partial, and within the limits of our conditions, it likely reflects a quicker recovery of the mice from the drug effect. This demonstrates that the time of measurement is important when assessing behavioral tolerance against a drug effect. Little or no tolerance to zolpidem has been reported in rodents (Sanger and Zivkovic, 1987; Perrault et al., 1992; Elliott and White, 1999). Sanger & Zivkovic found a small degree of tolerance to zolpidem, using a fixed-ratio lever press test in rats, a result that was never replicated (Sanger and Zivkovic, 1987, 1992). The only study conducted in mice, reporting no sedative tolerance, used very high doses of zolpidem (30 mg/kg, twice daily) and measured motor activity 42 hours after cessation of drug treatment, i.e. in a state of drug withdrawal (Perrault et al., 1992), whereas we tested the efficacy of the drug within the time frame of the chronic drug administration. However, in baboons, no difference was found between zolpidem and classical benzodiazepines with regard to tolerance to the ataxic effect (Griffiths et al., 1992). In humans, clinical trials did not reveal any tolerance after chronic zolpidem treatment (Voderholzer et al., 2001), however, case reports of tolerance to zolpidem are known (Sakkas et al., 1999; Aragona, 2000).

A first explanation for the reduced sedative effectiveness in mice chronically treated with zolpidem is a faster turnover of the ligand. However, it is unlikely that this early recovery from the sedative effect of zolpidem results from a change in drug metabolism, because no pharmacokinetic change has been reported with chronic zolpidem administration (Trenque et al., 1994) or chronic classical benzodiazepine treatment (Hutchinson et al., 1996).

Secondly, it has been suggested that a mechanism involved in the development of tolerance might be endocytosis of cell-surface $GABA_AR$ (Tehrani and Barnes, 1997; Ali and Olsen, 2001). In transfected cells and rat brain α1-containing $GABA_AR$ are internalized after chronic benzodiazepine treatment. The sedation produced by the test dose of zolpidem implies that no internalization of α1-$GABA_AR$ had taken place, which is in line with the sedation observed in α5(H101R) mice after chronic diazepam treatment (Fig.1c). If endocytosis of $GABA_AR$ played a major role in the development of this partial tolerance with chronic zolpidem treatment, the reduction of its motor depressant efficacy should have been evident immediately after the test injection, which is the case with chronic diazepam treatment. We demonstrated here that the sedation of zolpidem *per se* is not affected, only its duration.

It was recently shown that the acute motor depressant action of zolpidem depends exclusively on the activation of α1-$GABA_AR$ (Crestani et al., 2000, personal communication from F. Crestani). Therefore a third possibility is that the shortened sedative efficacy of zolpidem may be the manifestation of a change in the α1-$GABA_AR$ system occurring during the course of the chronic drug treatment. This could for instance be adaptations in the channel desensitisation kinetics, or changes in receptor recycling to the membrane. There also remain possibilities that extrasynaptic receptors could be activated by GABA spillover, affecting the α1-mediated inhibition. We, also, cannot exclude that the influence of associative factors in relation to the drug-associated context, i.e. resetting of the context-specific changes in synaptic plasticity, in the expression of sedation during the course of the chronic zolpidem treatment regimen, as this was proposed for sedative tolerance to diazepam (Marin et al., 1999).

We showed before that no major change occurs on total benzodiazepine binding sites, and another study has demonstrated that α1-binding sites are not changed even after 3 weeks of diazepam administration (Wu et al., 1994). Furthermore, neither acute nor chronic zolpidem administration reduced the number of α5-$GABA_AR$ binding sites as seen in diazepam-tolerant animals (Fig 2). In keeping with its low affinity for α5-$GABA_AR$, this result indicates that the reduction of the sedative efficacy of zolpidem with chronic treatment is independent of any change in these receptors. Thus, the mechanism of action underlying the chronic behavioral effect of zolpidem differs from that involved in sedative tolerance to diazepam. Chronic augmentation of only α1-$GABA_A$ receptor-mediated inhibition apparently is not sufficient to trigger down-regulation of α5 $GABA_AR$. However, chronic diazepam treatment of α1(H101R) mice, with drug binding only to α2-, α3- and α5-containing $GABA_AR$, also does not result in reduction of α5-binding levels (van Rijnsoever 2004, previous chapter). As α2- and α3-$GABA_AR$ are not involved in sedation or tolerance to the sedative drug action, this implies that a tolerance-associated reduction in α5-

$GABA_AR$ binding sites requires concomitant benzodiazepine binding to α1- and α5-$GABA_AR$ subtypes. Interestingly, diazepam treated animals tested with zolpidem also did not show any change in α5 binding sites. The last drug binding to α5-$GABA_AR$ was, at the time of sacrifice, 23 hours ago. α5 Binding levels were reported to return to basal levels 2 days after stopping a 4-week flurazepam treatment in rat. In the same study, the α5 binding levels also returned to normal after ending a 3-week diazepam treatment, albeit on a slower timescale (Li et al., 2000). The normal α5-binding levels observed in our mice could thus be due to lack of drug interaction with the α5-$GABA_AR$ subtype. However, since we did not test diazepam-treated animals with vehicle, we cannot exclude the possibility of a reversal by zolpidem of the α5 binding site downregulation associated with diazepam tolerance (Zanotti et al., 1999).

In conclusion, these data confirm that drug-induced enhancement of phasic GABAergic inhibition mediated by only α1-$GABA_AR$ is insufficient to induce full sedative tolerance. A likely partial adaptation to the sedative effect occurs, which does not involve any change in the number of α5-$GABA_AR$ binding sites. This might occur through adaptive mechanisms. When benzodiazepines bind to α1- and α5-$GABA_AR$, perhaps through the concurrent reduction in α5-$GABA_AR$ binding sites, full sedative tolerance will be displayed.

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4 General Discussion

In this thesis we used two strategies to investigate the regulation of $GABA_AR$ under normal conditions and after diazepam exposure. We assessed constitutive short-term regulation in cultured neurons. Furthermore, we examined the $GABA_AR$ subtypes that contribute to the development of sedative tolerance to diazepam.

In the first work we demonstrated that in hippocampal cells constitutively endocytosed $GABA_AR$ are relocated to a pool located within the postsynaptic density. We conclude this from the observations that 1) the membrane needs to be permeabilised before the postsynaptic clusters can be detected, 2) the endocytosed clusters appear in a time-dependent manner and 3) that their formation is inhibited when clathrin-coated vesicle mediated endocytosis is prevented. This intracellular pool might be involved in short-term regulation of $GABA_AR$.

The major finding from our second study using point-mutated mice was that tolerance to sedation requires specific interaction of diazepam with both the α1- and the α5-$GABA_AR$ subunit, without any involvement of α2- or α3-containing $GABA_AR$. Changes in α5 binding sites associated with sedative tolerance were also only seen when both α1- and α5-subtypes were available for diazepam binding. This was confirmed by the results obtained in wild type mice chronically treated with the α1-selective ligand zolpidem, which only displayed a partial sedative tolerance without any reductions in α5 binding sites. Thus, α1- and α5-$GABA_AR$ subtypes in particular mediate the development of sedative tolerance to diazepam.

Below I will discuss the two lines of investigation and argue whether the constitutive mechanisms found in the first study could play a role in this sedative tolerance to diazepam.

Constitutive $GABA_AR$ localisation and regulation in neurons.

The effect of benzodiazepines is governed by the α–subunit of the $GABA_AR$, with α1- and α2- $GABA_AR$ subtypes mediating sedation and anxiolysis respectively. In the hippocampus, α-subunits are expressed in different cell types (Fritschy and Brunig, 2003): the α2 subunit is mainly found in pyramidal cells, highly concentrated on the axon initial segment of cortex and hippocampal neurons and some clusters on the soma-dendritic compartment. The α1 subunit is highly expressed in hippocampal interneurons, but is also found at the soma and dendrites of hippocampal and cortical pyramidal cells. Although expressed in different cell types, the subcellular distribution of α1- and α2- $GABA_AR$ observed in hippocampal cell culture is very similar, since α1-subunits on interneurons and α2-subunits on pyramidal cells are both localized at postsynaptic sites (Brunig et al., 2002a). Furthermore, we demonstrated that both the subunits accumulate at postsynaptic sites after internalization where they colocalize with gephyrin. In contrast to α1- and α2-

subunits, the α5-$GABA_A$ receptor subunit is mainly extrasynaptic and does not cluster at postsynaptic sites (Crestani et al., 2002). Efforts to investigate whether α5-subunits are also endocytosed constitutively were unsuccessful. However, since no obvious clustering of α5-subunits is detected in a normal staining, it seems unlikely that endocytosed α5- $GABA_AR$ accumulate in an intracellular pool as observed for the α1- and α2-subunits. Thus, this intracellular pool colocalized with gephyrin might be a feature specific for subunits that cluster at synapses.

The constitutive endocytosis of $GABA_AR$ subunits is distinctly different from that observed with epifluorescence microscopy for the GluR1 subunits, which probably reflects the differential function and regulation of these two receptor types. First, GluR1 endocytosis is induced within minutes by agonist stimulation of AMPA R or NMDA R (Carroll et al., 1999; Lissin et al., 1999). This agonist-induced endocytosis of AMPA R allows for fast changes in synaptic strength and it is thought to be one of the mechanisms contributing to the development of LTP and LTD. In contrast, GABAergic transmission rarely seems to influence the localisation and constitutive endocytosis of $GABA_AR$. In primary neuronal cultures mismatched clusters ($GABA_AR$ opposed to glutamatergic terminals) are observed (Brunig et al., 2002b) and blocking synaptic transmission had no effect on $GABA_AR$ clusters during synaptogenesis (Craig, 1998; Studler et al., 2002), evidence that synaptic clustering of $GABA_AR$ is independent of GABA activity. The lack of effect of regular GABAergic transmission on $GABA_AR$ localisation is consistent with our observations that under normal conditions, accumulation of internalized $GABA_AR$ at postsynaptic sites is not influenced by blockade or acute enhancement of GABAergic activity. Although increased internalization after 1-4 hours of GABA exposure at 37°C has been shown (Tehrani and Barnes, 1997) and 48 hours of GABA resulted in downregulation of $GABA_AR$ in whole brain neuron cultures (Lyons et al., 2001), these exposures to GABA most likely do not happen under physiological circumstances.

Another point in which $GABA_AR$ distinguish themselves from AMPA R, is their specific relocation to the synapse after their endocytosis, with very little clusters found within the cell body. Stimulation of AMPA R usually results in the rapid accumulation of GluR1 subunits in the soma of the neuron (Carroll et al., 1999; Lissin et al., 1999; Beattie et al., 2000). Over an hour after agonist exposure, most of these subunits are recycled again into the plasma membrane (Ehlers, 2000). However, although these subunits are able to return to the synapse again when degradation is inhibited, these endocytosed subunits lack the clear synaptic pattern observed with $GABA_AR$ subunits.

It is not surprising that GABAergic transmission hardly affects the $GABA_AR$ localisation, whereas AMPA R respond so quickly and profound to glutamatergic transmission. The increased Ca^{2+} levels resulting from NMDA R opening can activate several proteins, such

as PKC or CamKII, whereas proteins that react to increased Cl^- influx are not known at present. In contrast, *indirect* modulation of $GABA_AR$ can be rapid and involves receptors that can activate PKA, PKC or tyrosine-receptor dependent signaling mechanisms. Changes in $GABA_AR$ surface expression can be induced within 10-30 minutes via modulation through the following types of receptors: Activation of muscarinic achetylcholine receptors (Brandon et al., 2002b) or 5-HT_2 receptors (Feng et al., 2001) reduced the GABA-activated response via a RACK1-PKC mediated mechanism. Stimulation of insulin, on the other hand, rapidly increases surface $GABA_AR$ (Wan et al., 1997), possibly through activation of tyrosine kinase (Brandon et al., 2002a; Ma et al., 2003). Thus the intracellular subsynaptic $GABA_AR$ that we detect might provide a way for fast regulation of $GABA_AR$ after modulation via phosphorylation-dependent mechanisms activated by other types of receptors. Adaptations through these second messenger pathways could possibly contribute to long-term regulation of $GABA_AR$, e.g. during the development of tolerance to benzodiazepines.

$GABA_A$ R regulation in the brain by benzodiazepines

The several $GABA_AR$ subunits are not only expressed in different cell types, they also have their own subtype-specific localisation in certain brain regions. On a global level, α1-subunits are most abundant in the cortex, α2-subunits in striatum and hippocampus, α3-subunits in olfactory bulb and cortex and the α5-subunit is highly enriched in the hippocampus (Fritschy and Brunig, 2003). The major finding from our second line of investigation is that tolerance to sedation requires interaction of diazepam with both the α1- and the α5-$GABA_AR$ subunit. The molecular change associated with sedative tolerance, a reduction in α5 binding sites in the dentate gyrus, appears only with diazepam, which was able to bind to α1- and α5-$GABA_AR$. It is unclear, however, whether this decrease is a cause for the restored motor activity or a consequence of adaptive, compensatory mechanisms that underlie the state of tolerance. It is known that tolerance to various effects occurs on different timescales: whereas tolerance to sedation usually develops within a week, tolerance to the anxiolytic effect requires at least 3 weeks of treatment (Hutchinson et al., 1996a). It is therefore likely that different $GABA_AR$ subtypes are involved in the development of tolerance to sedation and anxiolyis. It will be interesting to determine whether α5-$GABA_AR$ are always involved.

Several hypotheses have been suggested to explain the development of tolerance. One of the first was the idea of a ‘shift’ in benzodiazepine action, in other words, that chronic benzodiazepine treatment results in intrinsic changes in the $GABA_AR$ so that a full agonist would act as a partial agonist or even an antagonist (Stephens, 1995). This has been refuted by the fact that full allosteric agonist treatment does not result in similar amounts of cross-tolerance (Rosenberg, 1995). Moreover, our results show that it is the $GABA_AR$ subtype

interaction, rather than the full agonist action, that determines the development of sedative tolerance.

A second possibility that has been considered is a change in subunit composition: the total number of α1-binding sites does not seem to change after diazepam exposure (Wu et al., 1994a); however, changes in mRNA and protein expression have been reported (Hutchinson et al., 1996a; Barnes, 2000; Costa et al., 2002; Biggio et al., 2003). These reductions in protein or mRNA expression could also be related to the drug used, as changes are often reported with flurazepam (Zhao et al., 1994; Tietz et al., 1999a; Tietz et al., 1999b). Changes observed after diazepam exposure are often with either high doses or treatments of 14 days or more (Wu et al., 1994b; Holt et al., 1996; Impagnatiello et al., 1996; Pesold et al., 1997). Recently, it was shown that this reduction in α1-mRNA is specifically located within the dendrites, implying a local regulation mechanism for $GABA_AR$ expression (Costa et al., 2002). Interestingly, α1-specific ligands are devoid of effects on mRNA or protein expression. A decrease in α1-subunit mRNA was detected in cultured cerebellar granule cells after 5 days diazepam exposure but not after zolpidem (Biggio et al., 2003). Although changes in receptor subunit mRNA and protein expression have been demonstrated, it seems unlikely that these changes contribute to the sedative tolerance to diazepam observed in our mice. First, with the doses used in our study, we failed to detect any effects on total numbers of benzodiazepine binding sites. If changes in α1-subunit protein expression would occur (Impagnatiello et al., 1996; Pesold et al., 1997; Chen et al., 1999), diazepam-tolerant α2(H101R) mice should reveal a change in binding sites as well. Interestingly, even the point mutation did not unmask any reduction in binding sites that might go unnoticed in wild type mice. Therefore we can rule out any major effects of diazepam on α1- or α2- containing benzodiazepine binding sites in the regions investigated. In the end, we could only detect a reduction in α5 binding sites in specific regions with a specific α5 ligand. Second, the motor activity of diazepam-tolerant wild type mice was still reduced by the α1-specific ligand zolpidem, implying that, even if α1-$GABA_AR$ subunit expression was reduced, this was not enough to result in loss of zolpidem's sedative capacity. Last, an acute injection of the antagonist flumazenil is known to readily reverse the tolerance (Tietz et al., 1999b). Since the trafficking of newly assembled $GABA_AR$ from the Golgi to the membrane takes at least 6 hours (Gorrie et al., 1997), it is unlikely that changes in receptor subunit composition are the main cause for the development of tolerance.

A third explanation is that the loss of allosteric coupling between the GABA-binding site and the benzodiazepine-binding site of $GABA_AR$ is lost after chronic benzodiazepine exposure. This is called uncoupling (Klein et al., 1994; Friedman et al., 1996). The uncoupling hypothesis is in line with a fourth idea, that tolerance to benzodiazepines might be caused by receptor internalization (Tehrani and Barnes, 1997; Ali and Olsen, 2001;

Bateson, 2002). As uncoupling is measured using membrane-permeable benzodiazepines and the membrane-impermeable GABA, it is logical that internalization of $GABA_AR$ will result in uncoupling, since the benzodiazepines can bind to intracellular receptors whereas GABA cannot. The presence of an intracellular pool of $GABA_AR$ directly under the synapse would provide the possibility to quickly recruit $GABA_AR$ to the membrane e.g. after flumazenil exposure. Autoradiography does not distinguish between surface and intracellular receptors, as the ligands we used are membrane permeable. A lack of benzodiazepine binding site reduction would not rule out a redistribution of $GABA_AR$ in diazepam-tolerant animals. Although $\alpha1$-$GABA_AR$ endocytosis might occur during chronic benzodiazepine treatment, this is unlikely to result in full tolerance, as the $\alpha1$-$GABA_AR$ were still pharmacologically active in diazepam-treated animals.

Since none of these hypotheses for tolerance explain our results, we propose that the development of tolerance to the sedative action of diazepam is not due to a loss of $GABA_AR$ function, but to adaptive mechanisms, as discussed in the next section.

Role of network and cellular adaptive mechanisms for the development of tolerance to diazepam

Our results show that the function of $\alpha1$-$GABA_AR$ is maintained in diazepam-tolerant animals, and that behavioral tolerance is accompanied by a reduction of a5-binding sites, which occurs only if the animals are treated and challenged with a ligand, such as diazepam, that activates $\alpha5$- $GABA_AR$. These findings suggest that signaling through $\alpha5$-$GABA_AR$ is important for the induction and 'maintenance' of the tolerance state. Since these receptors are mainly extrasynaptic, a chronic enhancement of tonic inhibition might be an essential component of the development of tolerance. The reduction of $\alpha5$- $GABA_AR$ in the dentate gyrus might represent one of these compensatory mechanisms. Indeed, the hippocampal formation is an important gateway interconnected with most of the neocortex. A reduction of $\alpha5$-binding sites in the input area of the hippocampal formation is therefore likely to maintain a high level of activity at output stations (Chagnac-Amitai and Connors, 1989), in spite of the repeated enhancement of $\alpha1$- $GABA_AR$ function by diazepam. Further adaptive responses are likely to occur in both the hippocampus and neocortex, as specific changes in NMDA R expression (Perez et al., 2003) and LTP threshold (Marin et al., 1996) have been reported after chronic benzodiazepine treatment.

Although adaptations at the network level might be involved in the molecular and behavioral expression of tolerance to sedation, other mechanisms cannot be excluded. For instance, since tolerance to the anxiolytic action of diazepam requires longer chronic treatment protocols, an important question raised by the present results is whether tolerance to anxiolysis is mediated by the $\alpha2$-$GABA_AR$ and how it develops following the network adaptations which occur during the first week of treatment. The expression and regulation

of different $GABA_AR$ subtypes might be under control of distinct mechanisms that would be activated only after prolonged treatment. Recent research on 5-HT_{1A} receptor- knockout mice revealed that these mice have an anxious phenotype. These mice were benzodiazepine-resistant, in other words, benzodiazepine treatment did not relieve the anxiety. It turned out that the expression of α2-$GABA_AR$ in the amygdala and cortex of these mice was reduced by 50% (Sibille et al., 2000). Thus, expression of $GABA_AR$ in amygdala is under the control of 5-HT receptors, likely via a PKA or MAPK signaling pathway. It is therefore conceivable that tolerance to anxiolytic action of diazepam involves a region-specific down-regulation of α2-$GABA_A$ receptor under the control of the latter signal transduction pathways

Furthermore, the α1-$GABA_AR$ can be modulated by CaMKII, which causes increases in phosphorylation and allosteric-modulator binding to $GABA_AR$ (Churn et al., 2002). It is speculated that CaMKII might be able to activate dormant, or intracellular receptors. Furthermore, changes in CaMKII are implicated in chronic diazepam treatment. Furthermore, changes in CaMKII expression have been demonstrated following diazepam treatment of wild type mice, but not in α1(H101R), which did not display sedation or sedative tolerance (Huopaniemi et al., 2004). Interestingly, CamKII is not expressed in interneurons (Sik et al., 1998), which are known to have the highest expression of α1-$GABA_AR$ subunits (Gao and Fritschy, 1993), suggesting differential, cell-specific changes in α1-$GABA_A$ receptor function after chronic diazepam tolerance. Even in principal cells, regulation of the GABAergic response after PKC or PKA activation is differential depending on the brain region, as PKC reduces mIPSCs in CAI of the hippocampus, but increases them in dentate granule cells (Poisbeau et al., 1999). These alterations after chronic diazepam treatment are not incompatible with an overall preservation of the function α1-$GABA_A$ receptors, demonstrated by our results. They imply, however, that chronic diazepam treatment might differentially influence their role in principal cells and interneurons to produce the behavioral effects measured in the present study.

Involvement of short-term mechanisms in long-term regulation of $GABA_AR$

As highlighted in the introduction, the cell-surface stability of $GABA_AR$ subunits depends on the phosphorylation of specific residues in the several $GABA_AR$ subunits. This provides an intricate mechanism for regulating the amount of surface and intracellular $GABA_AR$ on a short-term basis. This raises the question whether a short-term mechanism such as phosphorylation is implicated in the development of tolerance. It is has been shown that chronic benzodiazepine treatment increases the activity of PKA in hippocampus and that blocking PKA activity in a heterologous system results in an increased endocytosis of $\alpha1\beta2\gamma2$ $GABA_AR$. How chronic benzodiazepine could change these second messenger pathways is unknown, however, changes in the balance of phosphorylation activity could

influence the GABAergic tone by its effect of cell-surface $GABA_AR$. However, considering the involvement of adaptive mechanisms described above, it is likely that increases in phosphorylation activity have an effect on other types of receptors, such as the AMPA R, as well. This might influence the number of cell-surface of AMPA or NMDA R, resulting in changes of excitatory transmission that could counteract the drug-increased inhibition.

Conclusions & outlook

This study shows that there is an intracellular pool of $GABA_AR$ within in the postsynaptic density that could provide means for short-term regulation and, furthermore, that the α1- and α5-$GABA_AR$ subtypes contribute to the development of sedative tolerance, possibly by activating adaptive mechanisms. Endocytosis of $GABA_AR$ was suggested as a mechanism for tolerance to benzodiazepines. The constitutively internalized $GABA_AR$ might stay in the intracellar pool within the postsynaptic density after chronic diazepam exposure. However, our results imply that endocytosis of α1-$GABA_AR$ is unlikely to be the main cause of tolerance, as diazepam-tolerant mice are still sedated by a single dose of zolpidem. The specific, concurrent involvement of α1- and α5-$GABA_AR$ suggests that the increased tonic inhibition via α5-receptors plays an important role in the development of sedative tolerance. More research is needed to answer some of the open questions of this thesis:

1) We could not demonstrate any effect of acute application of agonists, antagonists or allosteric modulators on dynamic regulation of $GABA_AR$ subunits. It will be of importance to see with a biotinylation assay whether chronic administration of these compounds will be able to alter the rate of endocytosis or insertion in neurons.

2) We demonstrated the role of α5-$GABA_AR$ in the development of sedative tolerance. At the present moment, very little is known about this extrasynaptic receptor. It will be important to know if α5-$GABA_AR$ display the same constitutive regulation and endocytosis as seen with the synaptically localized α1- and α2-$GABA_AR$. One intriguing question is what the mechanisms downstream of the α5- $GABA_AR$ are that in the end result in sedative tolerance.

3) Although internalization of $GABA_AR$ is no major contributor to sedative tolerance, a basic question that needs answering is how phosphorylation influences constitutive regulation of $GABA_AR$ subtypes. This has partly been assessed by the use of phospho-specific antibodies for β subunits in a biotinylation assay (Jovanovic et al., 2004). As α-subunits do not contain any sites for PKC or PKA phosphorylation, internalization of these subunits could be investigated with the biotinylation assay with PKC and PKA activators. Furthermore, it would still be interesting to see if chronic benzodiazepine exposure increases phosphorylation of specific $GABA_AR$ subunits, or if PKC/PKA activity is higher.

In conclusion, our data show that there are distinct pathways for short-term and long-term regulation of $GABA_AR$. These data further our understanding of the dynamic regulation of $GABA_AR$ under constitutive conditions as well as in response to chronic diazepam treatment.

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EVERY HAPPY END

IS

A GOOD BEGINNING

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I WOKE UP THIS MORNING

AND FOUND THE WOLRD

ON MY DOORSTEP

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